

Why the recession is still here

Last week's meeting of the grandest finance ministers sought with rhetoric to conjure recovery from recession from thin air, in the face of all the evidence that recovery will be long postponed.

FINANCE ministers, the people who decide what taxes we pay, behave even more curiously as a group than singly. The meeting of the finance ministers of seven industrialized nations of the world (known as G-7 for 'Group of Seven') in advance of the annual meeting of the International Monetary Fund (IMF) in Washington last week, illustrates the point. Of course, the finance ministers were more than usually alarmed. Each has a keen sense of his or her government's political and fiscal problems, and was seeking last week a collective decision and declaration that would make the problems go away. Most governments are worried by the two-year pause in economic growth in North America, Western Europe and Japan. But the German government has other worries, mostly stemming from the decision at the end of 1989 to extend the prosperity of West Germany to the 17 million people of the old German Democratic Republic, with negligible economic wealth of their own. That the finance ministers eventually won a declaration is not surprising; they usually do. But this year's is more devoid of content than ever.

That is not surprising. The yearning for a return of economic growth is widely shared, not only by governments but by their electors. But economic growth cannot be conjured out of thin air, and is unlikely to be so conjured in the years immediately ahead, at least while governments also rightly set their faces against inflation. For one thing, the unprecedented volume of private debt left over from the booming 1980s means that people who find they have a little extra cash are more inclined to use it for paying off debts than for running riot in the shops. For another, the past few years have been memorable for the scale on which supposed wealth has taken to vanishing into thin air. Every bank that writes off \$1,000 million every quarter or so to cover bad debts, every company that goes bust and every office mega-building left vacant for months on end represents an investment of resources that will not now be required. Is it any wonder that what is called the 'recovery' is so long delayed and so fitful?

There is even worse to come, typified by Germany's problem. Offered the unexpected chance of German unity in 1989, the then West German government could not have turned it down. It is also unthinkable that it could have declined to treat the eastern *Länder* as if they were not from the outset part of an integrated state; the inevitable mass exodus from East to West there would otherwise have been would have been less serious than the political incivility that would have followed. So Germany has been borrowing heavily (an estimated \$40,000 million last year) and levying heavy taxes

(heavier than elsewhere in the European Communities) on those in jobs. One result of that is the battle joined last week between public-service workers and the federal government over pay increases for the year ahead. Another is the traditional manifestation of the readiness of the Free Democrats to abandon the Bonn coalition when the going gets tough.

The G-7's comment on this daunting situation, from which much that is unwelcome everywhere may yet flow, is a curious blend of insensitivity and lack of realism: German interest rates are too high, are impeding recovery elsewhere, and should be reduced by increasing German taxes still further. The remedy proposed is unrealistic because it is politically impossible. The manner in which it is advocated is insensitive because it is tantamount to saying that German unification is a matter for Germany alone, which is not what the rest of Western Europe believes.

There may be even worse to come because the costs of German reunification will be multiplied many times in future dealings between the West and Eastern Europe and the Commonwealth of Independent States. Last week in Washington, the Group of Ten (which has eleven governments as members) agreed to allocate \$6,000 million to a fund to stabilize the ruble; that should not be a direct cost unless all the dollars end up as still-worthless rubles. But the scale of general assistance to the East, running at at least \$25,000 million a year, is likely only to increase in the years ahead and consists of a transfer of real resources. The West's self-interest is the strongest reason why this wealth should be transferred, but it is akin to asking that water should spontaneously run uphill that there should also be an inflation-free restoration of economic growth in the West.

That is why the recovery for which G-7 was yearning last week is likely to be further delayed. A decade from now, things may be different, but even that is not certain. The best hope, for Western Europe especially, is that dealings with the impoverished East (as with the poor countries of the developing world) should concentrate on opportunities for increasing trade rather than on transfers of wealth. Sadly, the European Communities have so far dealt with the newly independent governments of Central Europe as if they are as much to be feared as upsetters of existing economic arrangements as are producers of lamb in New Zealand or of beef in Australia. Yet, for all the inconveniences it would bring, free trade with the East is the quickest way of stopping the drain of resources to the East, and thus of allowing economic growth again. That is what G-7 should be talking about when next it meets. □



Journals as policemen

Up to a point, the US Congress may be able to enlist the help of journals in cleaning up the record of research.

SOME wag in the US Congress has hit on a novel way of regulating the conduct of the scientific journals: let them toe the line, or their contents will be indexed separately or not at all by the National Library of Medicine and thus not retrievable through the many databases the library compiles and maintains. The tortuous origins of this proposal are described on page 7 of this issue. The question of why there should be a line is simply answered; to eliminate fraudulent and otherwise suspect findings from the scientific record. But where the line is to be drawn is not answered by the bill, but will be decided by consultation between the National Institutes of Health's Office of Scientific Integrity (OSI) and the editors of journals.

At least for journals strong in modern biology, and especially for their contributors, the proposed sanction will be a powerful influence towards compliance. Among other things, the sponsors of the legislation apparently have it in mind that journals should be prepared to publish retractions of invalid research reports in a form that will ensure that the retraction is eventually mated bibliographically with the original. Thus stated, the plan is unexceptionable. Many journals (*Nature* included) follow such a course already. Others, mystifyingly, refuse to do so on the grounds that their standing might then be compromised. A little coercion would help mightily.

But what exactly is a retraction? First, even when made explicit, it is not necessarily a confession of dishonesty on anybody's part. Honest mistakes happen, as with last year's misinterpretation of pulsar data suggesting that there is a planet around a particular neutron star, and are as often as not retracted quickly and honestly. Will the US Congress allow that retractions, always painful for the authors, are not always marks of venality? And what is to be made of retractions that masquerade as reports of further and apparently successful research, as when an author reports results whose effect is to restrict the generality of a phenomenon previously reported? Or when an author follows a report in one journal with a report in a second journal that evidently supersedes the first? Such practices are probably more common than supposed, but journals cannot police them. Nor can journals confidently arbitrate in cases where, after publication, authors disagree about the validity of what they have published. (They can, of course, and should, report the disagreement.)

There are more serious dangers in what the Congress is brooding about. Few serious journals would cavil at the enforcement of a common policy on the form of retractions, but there would be much more serious doubts over policies to apportion credit for research results among the several authors of a joint research report or to compel the sharing of research materials with all enquirers (desirable though that may be). Journals with academic pretensions, knowing their

contributors to be mostly honest and meticulous, but knowing little of what Congress is up to, will be naturally less distrustful of the former than the latter. That is why close attention will be paid to OSI's negotiations with journals if this legislation becomes US law. □

Britain's new chance

The British government should not waste the opportunity it has given itself of restoring battered relations.

Is the British government, still glowing warmly after its unexpected election victory this month, able to seize the opportunity it has given itself to put British science in better shape? The decision to give a minister (Mr William Waldegrave) part-time responsibility for science is welcome in itself, but is also an opportunity for a fresh start. For far too long, the government's rejoinder to complaints at its handling of British science has been complacent denial. With a new organization, it can begin to make more intelligent responses.

On the administrative side, there is a difficult trick to perform. The designation of a minister with responsibility for science is the biggest organizational change since 1971, but the British scientific community is fed up to the teeth with reorganization, and needs a respite from it. How to marry these circumstances? By leaving the supposedly autonomous research councils, the chief sources of funds for academically related research, intact for the time being. Indeed, they all deserve a greater sense of responsibility within their terms of reference.

That argues for letting lapse the Advisory Board for the Research Councils (ABRC), whose chief function is to divide the annual budget for civil science between the research councils and the Royal Society, when its now full-time chairman, Sir David Phillips, retires next year. (Technically, ABRC gives advice only, but only contentious ministers such as Sir Keith Joseph reject it.) The truth is that ABRC has become a needless irritant to its dependants, more a part of the problem than of its solution. The research councils would probably be better off arguing their case with civil servants direct.

There is also a case for letting lapse the government's other chief advisory committee in the field, the Advisory Council on Science and Technology (ACOST), intended as a means of carrying out perceptive studies of important issues, but which has consistently failed to achieve its mission (perhaps for lack of adequate resources). This journal (see *Nature* 356, 644; 1992) would rather have an independent committee to act as a lightning rod and sounding-board for the opinions of the scientific community (malcontent and otherwise) and with the freedom to publish its reflections on them. But there is no great hurry. If this is to be a new beginning, it is better that the government should spend time building an organization that will last than that it should settle for another Kleenex institution, one destined to be discarded soon. □

NIH report vindicates Gallo on conduct of AIDS research

Washington. The US National Institutes of Health's (NIH) two-year investigation of alleged misconduct by two of its own scientists — AIDS researchers Robert C. Gallo and Mikulas Popovic — has now come close to the end with completion of a report by the NIH Office of Scientific Integrity (OSI) that, as with so many OSI reports that are distributed to principals and Congress, has leaked to the press.

The impetus for the OSI investigation was an article published in the *Chicago Tribune* by John Crewdson in November 1989, in which Crewdson implied that Gallo may have stolen the AIDS virus from French colleagues. Directly or by innuendo, the *Tribune* article also challenged the validity of some of Gallo's published work and criticized his behaviour generally in the race to identify the cause of AIDS.

The OSI report, written in January, clears Gallo of charges of misconduct but is nonetheless critical of the way he runs his laboratory at the National Cancer Institute. The report finds Popovic guilty of three instances of misconduct in writing the paper in which the Gallo laboratory reported successful growth of an AIDS virus isolate and it concludes that Gallo "breached his overall responsibility...as senior author to ensure the accuracy of the paper." While the OSI says it cannot "condone" Gallo's behaviour it also said the alleged breach of responsibility does "not constitute scientific misconduct."

The report is sternly critical of Gallo in many places and acknowledges that its authors did not always agree with one another on how to evaluate the evidence before

them. Thus, the actual findings of the report and its language are sometimes at odds.

The OSI finds now, as it did a year ago, no evidence to support allegations that Gallo stole or otherwise misappropriated the virus he used in the development of the AIDS blood test from his former colleague, Luc Montagnier of the Pasteur Institute in Paris.

The OSI, in its 114-page typed report, exonerates Gallo of misconduct in more than 15 counts of alleged data misrepresentation in a paper published in the 4 May 1984 issue of *Science* on which Popovic was principal author. In addition, the report notes that three other Gallo papers in the same issue (each with a different principal author) accurately report the experimental data — a point that contributed to the OSI's decision to put the blame for shortcomings in the first paper squarely on Popovic (see story below).

Further, OSI takes issue with Crewdson's interpretation of several events, generally taking the position that the evidence does not support a negative conclusion.

Defining scientific misconduct as "falsification, fabrication or plagiarism", the OSI has exonerated Gallo of virtually all charges against him. The report notes that one dissenter believed that a broader definition of misconduct should apply. According to the report, one unnamed OSI investigator believed that Gallo's oversight of his laboratory and attention to detail in the Popovic paper showed an "apparent disregard...for accuracy and responsibility in the conduct and reporting of research [that it] did constitute scientific misconduct."

This is the second version of the OSI report, the first having been rejected several months ago by NIH director Bernadine Healy more for its style than its content. She praises the new version for laying out the allegations and the evidence on both sides, as well as for putting the OSI analysis in clear focus. In substance, she says, the new version is similar to the first draft.

Representative John Dingell (Democrat, Michigan), who has the earlier draft, has already declared the revised report "watered down."

An additional report figures in the NIH's final disposition of the Gallo case. In an effort to ensure that the OSI's investigation was both comprehensive and did not favour NIH, former acting director William Raub created an *ad hoc* committee of advisers to act as a board of overseers. Never formally constituted as a government committee, the group was assembled under the chairmanship of Yale University crystallographer Frederic Richards.

Following transmission of OSI's final report to Healy in January, the so-called Richards committee submitted its own view of the case. Although the language of the OSI's report suggests in places that its exoneration of Gallo was somewhat grudging, the Richards committee was overtly critical of his conduct.

The committee accused Gallo of "intellectual recklessness", blamed him for inadequately describing the extent of his laboratory's study of the French virus and suggested that, by allegedly failing to share the

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OSI finds Popovic guilty on *Science* paper

Mikulas Popovic, the Czech-born chemist (now unemployed) who performed the crucial experiments in Robert Gallo's laboratory that resulted in the growth of an AIDS virus in sufficient quantity to develop a blood test, is guilty of three instances of misconduct. The OSI report focuses on the paper he published in the 4 May 1984 issue of *Science* describing his work.

■ Two tables in the paper includes the notation "ND", stated as meaning not done. However, analysis of laboratory notebooks showed that in each instance an immunofluorescence analysis of the "ND" cells had in fact been done. The OSI did not accept Popovic's rebuttal that he meant "'not determinable' because the findings did not make sense when compared to the other data..."

■ In reporting immunofluorescence assay results, Popovic wrote 10 per cent in a table, whereas the notebook kept by his technician reads "very few cells positive for rabbit antibody". OSI termed his data falsification, rejecting Popovic's claim that the 10 per cent figure was derived from his independent visual analysis of the cells.

■ One of the more unusual aspects of Popovic's experiment was a decision to pool several HIV isolates into a viral soup in an effort to get one of them to grow vigorously. A sentence in the *Science* paper says that the cultures used in the pool were "first" shown to have reverse transcriptase (RT) activity (indicating the presence of a retrovirus) is "false". Only one of ten samples was shown to be RT positive before being pooled.

According to OSI, the word "first" appeared in the seventh draft of the manuscript. Not until the eighth draft did it appear as a separate sentence. Popovic denies writing the sentence, although it is not clear who, among many editorial hands, did. However, OSI concludes that because Popovic bears overall responsibility for the paper, he is responsible for the error and, therefore, guilty of scientific misconduct.

In passing the OSI's report up the line, NIH director Bernadine Healy has recommended leniency, noting that the cited errors are not "material" to the validity of the paper whose findings have stood the test of time.

B.J.C.

(continued from page 3)

AIDS virus with colleagues once he and Popovic had successfully grown it in permanent cell lines, Gallo actually slowed the pace of AIDS research. Further, the committee faulted the OSI for its focus on the one *Science* paper in its final analysis of the case, arguing that by so doing it missed the forest for the trees in terms of assessing Gallo's professional behaviour.

The two reports put Healy in a quandary. If the Richards committee's view was fair, then the OSI's exoneration on strict definitional grounds might be misplaced, she reasoned, before doing what people in Washington do. Healy created a committee to advise her on the reports of the OSI and Richards committees: she convened the scientific directors of the 13 NIH institutes and gave them both reports.

Then, going one step further, Healy called Gallo before her own committee of advisers for three hours on the night of 23 March and challenged him, in effect, to rebut the allegations of the Richards committee. According to Healy and others who participated in the interrogation, Gallo did just that. In the words of one participant, "He blew the directors away, including those prejudiced against him."

Whether that is an exaggeration or not, participants agree that Gallo presented compelling evidence in his defence. For instance, on the serious charge of failure to share HIV with other laboratories, he presented files showing that the virus was sent to some 45 laboratories around the world in the months following the May 1984 paper in *Science*. (However, it is true that, because of a petty argument, Gallo was slow to give samples of HIV to his NIH colleague, Malcolm Martin, who subsequently suggested that the structural similarity between the Montagnier and Gallo isolates argued that Gallo's isolate HTLV-III was really the French virus in disguise.)

The Richards committee did not examine (nor, apparently, ask for) evidence to support its conclusions, relying instead on the OSI. Thus, it did not know the extent to which HIV had been distributed. At Healy's request, four members of the committee did meet with Gallo recently for 30 minutes but it was not a fact-finding occasion.

Taking the evidence altogether, Healy came down on Gallo's side in her letter of transmittal of the report to HHS authorities. Gallo, she says, has been absolved and, unless those higher up in HHS who have final responsibility for approving the report fail to approve it, the department should do everything it can to "restore Dr. Gallo's reputation." This is not to say that Gallo deserves any prizes for collegiality in science. But "if collegiality is the measure by which we evaluate good scientists", she says, "we might as well shut down right now".

One particularly thorny issue of collegiality centres on Gallo's role in editing a paper by Montagnier that was pub-

The OSI on Crewdson on Gallo

The OSI is frank in admitting that much of its investigation was driven by an article published in the *Chicago Tribune* by John Crewdson in November 1989. Although Crewdson's article presented no new facts, the way he wove together known information implied a pattern of behaviour that could add up to misconduct. The two examples given here are illustrative of the OSI's analysis of the Crewdson opus.

■ Throughout the AIDS dispute, the extent of Gallo's study of LAV, and his acknowledgment of that extent, has been an issue. In 1986, through a Freedom of Information Act filing, US attorneys for the French received a copy of a letter written in 1983 about electron microscopy of several HIV samples from Gallo's lab. The original letter included EM positive data suggesting that LAV was growing in two cell lines.

In the copy of that letter, as received by the French lawyers and subsequently read by Crewdson, all reference to LAV was deleted. According to the OSI report, "The 1989 *Chicago Tribune* article did not assert Dr. Gallo or one of his colleagues was responsible for the deletion, but there was an implication that this might be the case."

In fact, an investigation revealed that Gallo's copy of the letter did contain a reference to LAV and that the altered version had come from somewhere else. (Gallo has always seen this as evidence someone conspired against him.) Although the source has never been found, OSI concluded that Crewdson's "implication" could not be sustained by the evidence.

■ Crewdson hinted at misconduct on Gallo's part because data in conference proceedings from a presentation at a 1983 meeting in Cold Spring Harbor included information that had not actually been presented. "The implication of the *Chicago Tribune* article was that the Gallo et al. chapter [published in 1984 with updated information] ...overstated the [Gallo lab's] progress toward discovering the cause of AIDS. The inquiry team did not find convincing evidence for this assertion." In fact, most of the chapters were updated, including one by "Luc Montagnier et al." **B.J.C.**

lished along with papers by Gallo in the 20 May 1983 issue of *Science*. The manuscript, which Montagnier sent to the magazine via Gallo, lacked an abstract which Gallo offered to write. In addition, he suggested two editorial changes in the paper that have generated endless dispute.

The OSI's analysis of this episode is illustrative of the tone of the report. A sentence added to the paper said, "The DNA sequence of these and other members of the HTLV (human T-cell leukemia virus) family being compared." OSI concludes that the added sentence is "in line with the substance" of the initial version of the paper, but it goes on to say that "The mere fact of the addition...without the explicit, unequivocal agreement of the coauthors...[constitutes] a gratuitous, self-serving, and improper act on the part of Dr. Gallo."

The OSI report, however, does not stop there. In reaching its final analysis, it notes that because Montagnier received galleys of his manuscript, and could have objected to the added sentence if he wished, the editing by Gallo does not amount to scientific misconduct.

In an angry rebuttal to this (and other points), Gallo's attorney, Joseph Onk, argues that because it was Gallo who reviewed the manuscript favourably for *Science* and agreed to hold publication of his own paper until Montagnier's was ready, it is unfair to attack him. "Is this uncollegial?" he asks.

And so it goes.

The "final" OSI report is, sad to say, not the end of this tiresome story. Once the

report is cleared by her political bosses at HHS, Healy will argue for its open publication. Gallo's attorney, who along with Popovic's lawyer has written a rebuttal challenging points in the OSI report that impugn Gallo's behaviour, has said that if the report is made public, his rebuttal should accompany it. There will be plenty for Gallo supporters to like, in particular, the conclusion of "not guilty", and plenty for Gallo's critics to cite against him.

Meanwhile, Healy, angered by the unceasing accusations against Gallo, is arguing for an open administrative hearing to review the case yet again. She suggests bringing in an independent administrative law judge to run it. Referring to a recent nationally televised broadcast in which anti-Gallo parties to the dispute accused him of stealing or otherwise "misappropriating" the French virus, Healy says that "these people should come forward at an administrative hearing and present their evidence. On television they can say what they like. If they have evidence, I want to hear it."

Meanwhile, a dispute over distribution of royalties from the US AIDS blood test remains to be resolved. The French are asking for a greater share of the money in light of data uncovered this year that the virus both Montagnier and Gallo thought had come from a French patient known as BRU actually was contaminated, first in France and then in the United States, by a virus from another French patient known as LAI. But that's another story.

Barbara J. Culliton

Congress looks for methods to assess clinical research

Washington. Attempts by the US Congress to get the best health care for its money have made it a central, if unwitting, figure in a fierce debate between researchers over methods to assess medical data. And a new congressional report aimed at resolving some of the issues has instead only fanned the flames.

The report*, by the congressional General Accounting Office (GAO), advocates the use of a new method of meta-analysis — known as 'cross design synthesis' — to combine the results from clinical trials and medical databases. But by siding with those who would use statistics to make up for deficiencies in existing clinical trials and other biomedical data, the GAO report advocates a methodology that critics describe as shoddy science and statistical legerdemain.

Legislators are looking for ways to quantify therapies that work best and give the best value for money. In the absence of scientific consensus, they have turned to what is known variously as 'technology assessment' or 'outcome assessment'. In 1989, Congress created the Agency for Health Care Policy and Research (AHCPR) within the Department of Health and Human Services to carry out such studies. Its annual funding has risen steadily, and now stands at \$120 million.

But Congress is doing more than handing over the money. It has also sought to dictate which approaches should be used. On congressional order, the agency is spending more than \$60 million on studies that combine existing clinical trial results with databases of insurance company or hospital records (known as 'administrative claims data'), or on research into the statistical techniques themselves. By encouraging researchers to combine and reanalyse previously inconclusive clinical trials or to comb through existing computer databases, legislators hope that they can avoid paying for long and expensive clinical trials.

But that is heresy to many researchers. The idea that analysis of computer databases can replace careful clinical trials "is not serious science, it's serious fund raising", says Richard Peto of the University of Oxford.

Most researchers agree that meta-analysis, broadly defined as the statistical analysis and combination of other studies, is a useful and sometimes essential tool. It works best as a way to draw conclusions from randomized clinical trials that are not sufficiently convincing by themselves because of small sample sizes, design flaws or other problems.

But an increasing number of researchers are applying meta-analysis and other related statistical techniques to non-randomized

clinical trials, trials with known and unknown biases and even database studies. They believe they can 'adjust' for whatever defects exist in the studies. Several groups are developing computer programs that would automatically correct many of the statistical flaws.

The use of meta-analysis and database analysis is fostered by money from AHCPR. Eminent researchers such as Harvard University's Thomas Chalmers — considered the father of the clinical trial — think meta-analysis is essential to clinical research. Dozens of private groups, from the American Medical Association and the insurance industry to the office of medical applications of research at the US National Institutes of Health (NIH), are now using the two techniques in their own technology or outcome assessments (see *Nature* 351, 598; 1991).

But several cases have raised questions about the value of using old and flawed data. A 1990 study that compared two treatments of prostate disease — surgery and a relatively new trans-urethral method — found that patients who received the non-surgical treatment had a greater chance of dying within the next few years. But the cause of death, paradoxically, was rarely related to the prostate. A study published earlier this year revealed that, because the trans-urethral method was less physically traumatic, doctors tended to use it on their older and sicker patients; these patients were, of course, also the most likely to die within a few years. When the data were corrected for comorbidity before surgery, the differences in mortality disappeared.

In the case of Herbert Needleman, a researcher at the University of Pittsburgh, a debate over meta-analysis may go to court. His 1990 analysis of 12 studies suggested a clear link between low-level lead consumption and reduced intelligence in children. But two other researchers did their own meta-analysis of many of the same data and found no link. Because the researchers were unable to reconcile their scientific differences, the debate turned into allegations of misconduct and has led to investigations by the university and NIH (see *Nature* 356, 466, 9 April 1992).

Critics of both outcome and technology assessment say that such cases illustrate the risks of using flawed, incomplete or deceptive data. Researchers such as Salim Yusuf, head of a clinical trials branch in the US National Heart Lung and Blood Institute, and Earl Steinberg, director of the medical technology assessment programme at Johns Hopkins University, say that no amount of manipulation can extract trustworthy results from flawed data. And they are quick to

cite a maxim from the computer industry: "Garbage in, garbage out".

Rather than trying to make do with existing data, many researchers want to carry out new and better clinical trials. With backing from the congressional Office of Technology Assessment (OTA) and parts of NIH, these scientists are asking for money for cheap, simple and large trials. One such trial was the International Studies on Infarct Survival that looked at heart disease in more than 30,000 persons from three countries.

The debate has led some members of Congress to reconsider their support for meta-analysis. Later this year, the Senate Labor and Human Resources Committee is expected to ask the OTA, which has traditionally been critical of statistical assessment techniques in medicine, to evaluate the various methods. Meanwhile, the New York Academy of Sciences is planning a conference to discuss the need for large-scale clinical trials. Both sides in the debate agree that their goals are better trials and better statistical evaluation of existing data.

Christopher Anderson

* Cross Design Synthesis: A New Strategy for Medical Effectiveness Research, US General Accounting Office; 1992.

Visitors to China urged to protest

Researchers travelling to China who want to protest at the imprisonment of Chinese scientists can now do it by the book.

A New York-based human rights group has released a guide to putting pressure on the Chinese government on behalf of researchers who have been jailed for political activism. In the guide, the Committee to End the Chinese Gulag suggests ways to help some 65 scientists now confined.

One approach, it says, is to present conference organizers with a list of imprisoned researchers. Dedicating a paper to such a researcher or collecting signatures on a petition are other options, as is displaying a protest poster. Scientists can also request an appointment with government officials, although the chances of success are slim. The guide (which may be obtained by calling 212-972-8400) also describes how to obtain publicity for any protests, especially if they are disrupted by Chinese authorities.

China is hosting a growing number of scientific conferences in an attempt to improve its international image, including major meetings this year on the physics of semiconductors and on entomology. Some physicists are boycotting the meeting in protest. For those who attend, the guide advises caution. Last week, China detained and expelled seven European legislators and union leaders after they staged a brief protest in Tiananmen Square.

Christopher Anderson

Biotech companies rescue Italian vaccine institute

Munich. One of Italy's most renowned research institutes, on the brink of closing in 1990, has a new owner, a new name and a new mission.

The Sclavo Research Institute in Siena was purchased earlier this year by Biocine, a California company set up in 1986 as a joint venture between Ciba-Geigy and Chiron. The two companies paid 77,000 million lira (US\$62 million) to preserve the world-class vaccine research group at the institute, whose name now is the Immunobiological Research Institute Siena (IRIS).



The proud tradition of the Sclavo Research Institute carries on with a new name.

The companies view the acquisition as a quick way into the paediatric vaccine market. They are streamlining IRIS's research operations and intend to focus exclusively on vaccine development. They have already announced plans to fill 30 new academic posts.

The Sclavo institute was set up by Achille Sclavo, professor of hygiene at the University of Siena, who in 1904 made a serum against anthrax in his cellar. As a privately owned company, Sclavo grew eventually to 1,500 employees, including 100 scientists. It became world famous for its development of the pertussis toxin (whooping cough) vaccine.

But the institute fell on hard times in the mid-1970s, as public confidence in vaccines was shaken after claims of serious side effects, particularly neurotoxicity. The world vaccine market collapsed and Sclavo was taken over by the state-owned company Enichem to protect Italy's source of sera and vaccines. In the early 1980s, Sclavo became one of Italy's first centres for biotechnology, with the Italian government paying almost half of its annual budget of 15,000 million lira.

But by 1990 the company was in trouble again, reporting a loss of 20,000 million lira in the first six months of that year. Enichem was sold to the Italian pharmaceutical and broadcast group Marcucci, which decided

to discontinue its commitment to biological research (see *Nature* 346, 497; 1990).

Responding to national and international pressure to save the centre, the Italian government stepped in once more. Research minister Antonio Roberti blocked moves by Marcucci to dismiss researchers and guaranteed payment of about 60 per cent of the centre's annual operating budget. In exchange, the government is believed to have considered more leniently the company's requests to lay off more than 300 workers at other plants. (The government pays 80 per cent of the wages of those who are laid off.)

Biocine was set up to combine Chiron's expertise in genetic engineering and Ciba-Geigy's experience in immunostimulatory molecules. The improved safety of recently developed genetically engineered vaccines has restored confidence in vaccination programmes for children in the eyes of the public, and Chiron officials believe that the acquisition of Sclavo will give them an established entry into the burgeoning paediatric vaccine market. They have also promised to maintain the institute's tradition of basic research in immunology.

Dino Dina, vice-president of Chiron's vaccine programme, says that Sclavo was an attractive opportunity despite the company's poor commercial performance under Enichem. "There are a lot of excellent people in the labs, it was set up with state-of-the-art technology for producing recombinant vaccines and it has a manufacturing capacity that is crucial to our programme", he said. The plans to restructure the company include dropping projects, such as inflammation pharmacology, that do not relate directly to vaccine development. Dina is confident that this strategy, combined with the initial investment — the 30 new academic posts are being established immediately — will help IRIS through its current problems.

Two new products are expected to be marketed by IRIS by the end of this year. One is a vaccine against *Hemophilus influenza B*, a virus responsible for about 40 per cent of the meningitis cases in Italy. The second is a vaccine against recombinant pertussis toxin, which will replace the current whooping cough vaccine, produced from whole cell cultures, whose association with neurological damage has limited its general acceptance. The institute's director, Rino Rappuoli, was last year awarded the Paul Ehrlich prize for his work on this vaccine. IRIS is also developing vaccines against *Helobacter pilori*, a bacteria associated with gastric ulcers and tumours, papilloma virus and cholera.

Alison Abbott

Archaeopteryx fossil disappears from private collection

Munich. Palaeontologists in Germany are trying to unravel the mystery behind the disappearance of one of the world's most important fossils.

One of only six identified specimens of *Archaeopteryx lithographica*, all of which have been discovered in quarries in the Solnhofen area of Bavaria, has been missing since the suicide of its finder and owner, Eduard Opitsch, in February 1991 at the age of 91. The incident has once again demonstrated the vulnerability and inaccessibility of privately owned fossils.

Archaeopteryx is particularly important to evolutionary biologists because it is the most primitive bird known. It provides the major evidence in the theory that birds have evolved from reptiles.

The Maxberg specimen, so-called because it was exhibited in the Maxberg Museum near Solnhofen after its discovery in 1956, was removed from public display by Opitsch in 1974. Since then Opitsch, known locally as an eccentric, has not allowed any access to the scientifically vital fossil.

After the death of Opitsch, who owned and operated a quarry in the area, his relatives could find no trace of the two limestone slabs (one approximately 50 cm by 35 cm, and the second 40 by 35 cm, and each slab 12 to 15 mm thick) that contain the fossil. Peter Wellnhofer, of the Bavarian Palaeontological Museum in Munich, fears that the fossil may be put up for sale on the black market. "We want the scientific community to be on the lookout for this specimen", he says. Anyone learning of its whereabouts, he says, "should be cautious, but inform either us or the police".

Items of natural heritage, as opposed to works of art, are generally not well controlled in Europe. Although the neighbouring state of Baden-Württemberg has strictly enforced laws against the movement of cultural possessions across its boundaries, Bavaria has no similarly protective laws.

Although the Maxberg *Archaeopteryx* is a relatively fragmented specimen, its scientific and monetary value is high. The best preserved specimen, housed in London's Natural History Museum, has been valued at around £2–3 million (US\$3.5–5 million). But Angela Milner, head of the museum's section on fossil amphibians, reptiles and birds, says that a monetary value is meaningless. "There are so few specimens of *Archaeopteryx* that this scientific loss is just extremely sad".

Alison Abbott

Anyone with information about the fossil should contact P. Wellnhofer at Bayrische Staatssammlung für Paläontologie und historische Geologie, Richard-Wagner-Str. 10, 8000 Munich 2, Germany; or the police at Staatsanwaltschaft Ansbach, Promenade 4, 8800 Ansbach, Germany.

Bill would force journals to follow misconduct rules

Washington. Overlooked in all the celebration over last month's passage of congressional legislation overturning the US fetal tissue research ban were a few paragraphs in an accompanying bill that, if made law, would essentially force scientific journals to adopt federal misconduct guidelines.

The language, which was passed by the House of Representatives as part of a bill to reauthorize programmes at the National Institutes of Health (NIH), directs the National Library of Medicine (NLM) to develop guidelines "...to protect against publication of manuscripts with respect to which there has been scientific misconduct..."

Although the bill does not describe what those guidelines should say (NLM is supposed to develop them in consultation with the NIH Office of Scientific Integrity and journal editors), the House staff who wrote the language say that they wanted journals to retract published articles when they are shown to be associated with scientific misconduct. If a journal refused to follow the guidelines, it would be left out of the library's abstract database. For many journals, especially specialized publications with small circulations, not being included in the NLM database is tantamount to not existing at all.

These provisions have actually been in the House NIH bill for three years. But the bill was never taken seriously because it included language to overturn a ban on the use of fetal tissue in research that the president, George Bush, had promised to veto. Although the bill passed the full House last July, it was not until last month, when the Senate passed its own NIH bill by a margin large enough to defeat a presidential veto, that people started to look seriously at the language in the House bill.

Journal editors are disturbed by the idea

that Congress is telling them what to do. Daniel Koshland, editor of *Science*, says that his journal already has a policy to retract articles — with or without the approval of the authors — "if we are convinced they are wrong". *Nature* has a similar policy (see page 2).

Koshland believes that the House bill — including its core definition of misconduct as acts that "seriously deviate from the standards of conduct that are recognized within the scientific community" — is more than a little ambiguous. "It's so loose and vague that I don't understand how it could be enacted", he says.

Jerome Kassirer, editor of the *New England Journal of Medicine*, is concerned that federal guidelines would allow the government to intrude into an evolving system of self-regulation. Most universities already have misconduct guidelines, he says, and they alert journals to research that may be the subject of misconduct investigations.

Many medical journals have also adopted misconduct guidelines developed last year by the International Committee of Medical Journal Editors. George Lundberg, editor of the *Journal of the American Medical Association*, says that congressional efforts to develop similar rules "are unnecessary and stand a chance to do more harm than good". Federal guidelines are particularly inappropriate for non-US journals, he says: "By trying to legislate how [the NLM] should work in regard to journals outside the United States, the Congress is sticking its nose where it does not belong."

Later this month, the House and the Senate will meet in conference to work out the differences between the two versions of the NIH bill.

Christopher Anderson

Universities fear being left out by patent reforms

Washington. A US university technology transfer association told Congress last week that plans to harmonize the US patent system with the rest of the industrial world could leave academic researchers unable to patent many of their inventions. Without special protections for universities and independent inventors, it argued, the planned US move from a 'first-to-invent' system to a 'first-to-file' system would favour companies that can afford expensive legal and filing fees.

The United States is negotiating with European nations and Japan as part of an international patent treaty process that would create uniform rules in all the major industrialized nations (see *Nature* 356, 645; 23 April 1992). At a joint House-Senate hearing last week, legislators heard testimony from patent experts and industry and university representatives on two bills that would change US patent law along the lines of the expected treaty resolution.

Howard Bremer, a lawyer representing the Association of University Technology Managers, pointed out that the work of academic researchers is especially vulnerable to changes in the patent system. University inventions are usually based on basic research and tend to be 'seminal', he said. If precedence goes to the first to file, university researchers may be forced to submit patent applications before they know the full utility of their invention. Worse still, he said, they may decide they cannot afford to file at all.

Large companies often have the resources to file applications to cover any discovery, however minor. If universities are forced to file early on basic discoveries, companies could build on the invention and file for uses that might not have occurred to the university researchers.

The current US system allows university researchers to file for a patent as long as a year after they publish a discovery. Both the bills in Congress and the draft international treaty include a similar 12-month 'grace period' after publication, during which an inventor would still be able to claim a patent.

Bremer said that acceptance of such a grace period by the rest of the world would calm the fears of most US universities. US academics also favour that portion of the British patent system that allows for a low-cost 'provisional' patent application, to be followed with a larger fee if the inventor decides to pursue the patent. Such a clause, Bremer said, would give universities time to choose the inventions they want to pursue without risking their patent rights.

Christopher Anderson

Report urges software patent fix

Washington. A new report* recommends that Congress should intervene to help the US Patent and Trademark Office come to grips with the issue of patenting computer software.

Noting that no issue short of patenting life has confounded the patent office more than the blizzard of claims for everything from basic mathematical principals to the use of on-screen symbols, the congressional Office of Technology Assessment (OTA) recommends that Congress should consider changing the law to clarify or restrict the scope of software patent and copyright claims.

In the meantime, OTA recommends several more immediate measures, including a database of non-patented 'prior art' — algorithms, programs and concepts considered to be in the public domain or otherwise unpatentable. Giving the public access to the database would help to keep it up to date, OTA says, and would allow software developers to judge the quality of their claim without actually filing an application.

Christopher Anderson

Finding a Balance: Computer Software, Intellectual Property and the Challenge of Technological Change. OTA, 1992.

Renewable energy may fall off Britain's research agenda

London. British scientists working on renewable energy technology are awaiting a report that is expected to give their work a shot in the arm. That would be good news for a field whose future is clouded following the defeat in last month's general election of the government's main advocate of renewables, energy minister Colin Moynihan, and the dissolution of the Department of Energy.

Last August, Moynihan set up the Renewable Energy Advisory Group (REAG) following a commitment to do so by the Department of the Environment. At the time the group was said to be "spearheading the government's review of renewable energy", although since then there have been attempts to play down its importance.

The REAG was expected to deliver its report early this year, but the process was suspended because of the general election. It had already taken oral evidence from 18 organizations and written evidence from nearly 1,000 more, but has yet to reach any conclusions or recommendations.

No decision has yet been made by the Department of Trade and Industry (DTI), which has taken on most of the Department of Energy's duties, on whether the REAG report will be completed. Many energy commentators, including some who gave evidence to the REAG, fear that the work of the REAG will be buried.

However, other observers believe that a recent favourable report on renewable energy from the House of Commons Select Committee on Energy is an adequate com-

ment. But this report, essentially an all-party commentary on the government's activities connected with renewables, lacks the potential impact of the REAG report, which was initiated by a minister and conducted within the department responsible for energy.

The transfer of energy policy, including research, from the Department of Energy to the DTI has itself caused dismay. Energy previously had high-level representation in the cabinet through the secretary of state for energy, who commanded a number of ministers, including Moynihan. Now there is just one minister for energy, Tim Eggar, whose time is expected to be taken up with selling off the UK coal industry and regulating the other privatized energy industries.

Although the select committee report was optimistic about the prospects for renewable energy in Britain—it recommended that the target for the annual contribution of renewables to the electricity supply by 2000 be raised from 1,000 MW to between 3,000 and 4,000 MW—it attacked the government's handling of research and development into alternative energy technologies.

"In the past the Department [of Energy] has attempted to establish costs at too early a stage in a technology's development; to draw final conclusions from tentative assessments; and to place too little value on continuity of funding", the report said. "It is difficult to regard the history of renewable R&D funding in the United Kingdom as other than a history of volte-faces, premature judgements and plain errors."

Ian Mundell

Space station wins, but House critic says that he's lost it

Washington. While the planned space station Freedom was winning a vote of confidence last week, one of its harshest critics was announcing that he had had enough. Representative Bob Traxler (Democrat, Michigan) said he would not seek reelection next year after 18 years in the House of Representatives because he felt "powerless" to effect substantive change.

As chairman of the appropriations committee that determines the budgets of the National Aeronautics and Space Administration (NASA) and the National Science Foundation (NSF), Traxler was one of science's strongest supporters in Congress. But his efforts last year to kill the space station—and use the money to fund research throughout the government—was defeated on the floor of the House after the Bush administration lobbied hard in its support.

Last week, that coalition of supporters held firm, defeating by a margin of 254 to 159 a proposal to kill the station and recover most of the \$2,200 million that NASA intends to spend on it next year. The vote to authorize NASA's programmes suggests that the lure of jobs and the excitement of space—two issues that supporters dwell upon—are more persuasive arguments than an extensive lobbying campaign by professional societies for more useful research than that to be conducted on the space station.

Ray Bye, NSF's director of legislative affairs, called Traxler's decision to step down "a terrible loss for the agency and for science". Representative Louis Stokes (Democrat, Ohio) is expected to replace Traxler as the chair of the appropriations subcommittee, which includes the housing, veterans and environmental agencies as well as NASA and NSF. Stokes is known as a supporter of education, housing and minority issues.

A rash of retirements in both houses of Congress have claimed several other legislators who are active in science. Howard Wolpe (Democrat, Michigan), the chairman of the House science committee's investigations subcommittee, announced last month that he would leave at the end of his seventh two-year term. Wolpe reinvigorated the subcommittee and triggered recent investigations of the Superconducting Super Collider, NSF's manpower studies, the US energy research programme (see story at left) and NASA accounting procedures. Bye describes Wolpe as "our conscience". On the Senate side, Jake Garn (Republican, Utah), the ranking minority member of the NASA/NSF appropriations committee, a strong space advocate and the first legislator to fly on the space shuttle, has also announced his retirement.

Christopher Anderson

Congress laments politics of energy

Washington. An investigation by a US congressional committee has come to the unsurprising conclusion that politics, rather than merit and economic priorities, drives the US energy research programme.

At a hearing last week, the investigations subcommittee of the House of Representatives science committee released internal documents from the Department of Energy (DOE) that compared the agency's analysis of the relative merits of various energy research programmes with the amount of money it actually requested for those programmes for fiscal year 1993.

DOE's policy office ranked the programmes on the basis of their expected contributions to the goals of the National Energy Strategy—principally, reducing US demand for oil. Energy efficiency efforts came out on top across the board;

nuclear fission, fusion and fossil technologies were at the bottom. "Political sensitivities can be applied later," the head of the policy office wrote to the DOE secretary last July, "but you need to know, first, what seems to be right on the merits...."

However, after DOE applied those 'political sensitivities', the picture was essentially reversed. The group given the highest priority was cut by an average of 52 per cent, and the group given lowest priority was increased by an average of 45 per cent.

One project that can thank politics for its funding is the Superconducting Super Collider. The policy office ranked the project second from the bottom as a basic research priority. But DOE ignored its recommendation to trim \$100 million from next year's proposed budget.

Christopher Anderson

Less science, more policy at Amsterdam AIDS meeting

Basic research is giving way to policy at the Eighth International Conference on AIDS. Judging from the number of abstracts that have been received, basic science is down by 21 per cent from last year, and policy is up 53 per cent, organizers said last week.

The new figures reinforce the perceptions of many AIDS researchers that scientific exchange is giving way to discussions among activists and policy specialists. The meeting, to be held 19–24 July in Amsterdam, is expected to draw about 7,000 people, of whom only about half are researchers and clinicians. Nearly 5,000 papers will be presented, both in poster sessions and talks.

"The sheer size of the meeting has made it unsuitable for the kind of scientific exchange that a lot of us feel is useful," says Anthony Fauci, head of the AIDS programme at the US National Institutes of Health (NIH). Although he says the meeting does a good job of refocusing public attention on the issue, "from the standpoint of day-to-day scientist, I don't think it gives you the kind of scientific input you need. I don't go there expecting that I'll come back with really new insights."

Although clinical research is at about the same level as last year, epidemiological research is down by about 11 per cent. But Jonathan Mann, the conference organizer, says that the epidemiological research is

more rigorously scientific and less descriptive than in previous years.

Mann says the shift towards policy is both inevitable and desirable in order to draw attention to the social aspects of the issue. "Sometimes we lose sight of the need for breadth," he says. "If people are concerned only about the virus, there are other meetings at which they can learn more. But if they're concerned about the epidemic, this is the place to be."

A handful of sceptics who believe that human immunodeficiency virus has not been demonstrated to cause AIDS are holding their own meeting in Amsterdam next week to present theories that they say are being ignored by the main conference. But Mann says that the main meeting has always been willing to consider alternative theories, and would usually accept them if they provide new information. Fauci adds that the general subject of co-factors, such as superantigens that contribute to immunodepression, are considered to be an important area of research.

"But whether you need a special meeting [on the subject] is another matter," he says. "I think these other immunopathic events are well represented at the regular conferences." So far, the alternative conference has signed up fewer than 100 participants.

Christopher Anderson

More green for greener satellites

London. Representatives of environmental interests want to have a greater say in planning the missions of Earth-observing satellites and the instruments they will carry in return for paying a larger share of the cost of such satellites.

An international organization representing most of the countries that are active in space met in London last week at the request of the British prime minister, John Major, to discuss how cooperation could improve the contributions that satellite data make to environmental management. In talks with officials from international environmental agencies, the members of the Committee for Earth Observations Satellites (CEOS) agreed to strengthen ties between the space industry and users of environmental data.

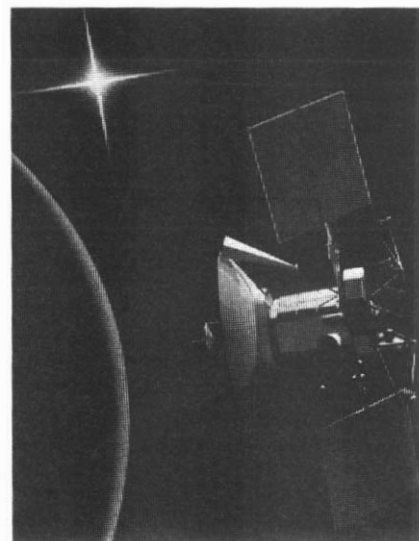
As major users of the satellite data, the environmental research community has been under pressure to contribute more money to the space industry. That need is likely to increase with the decline in support from military sources.

A dossier on existing and planned envi-

ronmental space observation served as the basis for the discussion. An extract of this dossier will be sent to participants at next month's Earth Summit in Rio de Janeiro to raise awareness of the importance of satellite missions. Meanwhile, another document to be compiled by environmental researchers for the next CEOS meeting in December will set out ways to make the best use of satellite data and to identify the needs of its users.

These moves may not put more money into satellite programmes, but they could affect the way in which each organization pays for its share of the cost, with a greater proportion coming from the environmental sector. The meetings are also expected to give greater prominence to CEOS, leading perhaps to a permanent office and secretariat. The committee, with representatives from most of Europe, Japan, the United States, Canada, India, China, Russia and Brazil, is run from whichever country holds the committee chair. Britain is in the chair for 1992.

Ian Mundell



Magellan team fights to extend mission

Washington. Project managers for the Magellan radar mapper are cutting costs and writing letters to keep the spacecraft alive. Although the project is scheduled to end its mapping of Venus by September 1993, project scientists say they could wring another 18 months of science from the probe by cutting operating costs in half. But to do that, they need at least another \$33 million next year and must overcome both a failing transmitter and the opposition of the National Aeronautics and Space Administration (NASA), which wants to end the project as planned.

"NASA's taking the position that we've already met our project goals and Magellan ought to be turned off", says James Scott, Magellan project manager at the Jet Propulsion Laboratory in Pasadena, California. But he and other project scientists believe that putting more spacecraft operations under computer control and cutting the staff from 270 to 170 will allow Magellan to operate at about half of its usual \$40 million annual cost.

NASA, however, wants to prove to Congress that it can keep to a budget, and has so far allocated only \$10 million for next year to complete the data analysis and produce a final report. Foiled internally, the Magellan team has now turned to outside pressure, appealing directly to members of Congress and to the vice president, Dan Quayle, who is head of the National Space Council.

But their chances are being hurt by the spacecraft's continuing technical problems. It is now operating on a backup transmitter, and even that has a failing part that is interfering with the data signal (see *Nature* 355, 190; 1992). Even with "heroic measures" by ground-based receivers, Scott says, Magellan's data signal is almost unusable. "If we had a better signal, we'd have a better chance at funding," he says.

Christopher Anderson

Science in Czechoslovakia

SIR — It is no secret that the young, mainly democratic governments of central Europe have huge economic difficulties. The national economies change from central planning to a market economy slowly, and along untrodden paths. Capital is lacking, and unemployment rises, while people feel victimized and discontented. In such conditions, cuts are made in 'nonproductive' areas such as culture, education, science and research, and in health care.

The defeat of totalitarian regimes offers a great opportunity for redressing injustice but, when democracy is neither established nor generally accepted, it is also an opportunity for social climbers and their shrewd practices.

Economic transformation logically affects the position of science in society, the financial resources for research institutes and the standard of living of particular researchers. Today, science is a Cinderella, and there are even voices saying that scientists and researchers are unnecessary. This could become very dangerous and the solution is not clear.

The 'velvet revolution' in Czechoslovakia has not ended. If people band together, regardless of their political inclinations, they can exert enormous pressure on science-related issues, particularly with one or two well-timed anti-communist speeches and a seat or chair in some committee in a research institute, in the academy or at an influential ministry.

Grant committees are a good example. The idea of competition for financial support has been tested in the West. But the Czechoslovak Academy, for example, both offers and approves of grants — they are sometimes nicknamed "family favours".

To defend this scandalous practice, the academy's advocates say that pure science is hard to justify in a state with serious economic restraints. When the publications of the research institutes of the academy were assessed, those receiving grants often lagged behind those whose grant applications were rejected. No wonder then that the Ministry of Finance is cautious about its financial contribution to the academy.

The chaotic situation also makes possible the creation of new institutes, with new directors. If it is asserted that large research institutes are not productive, a statement that cannot easily be disproved, one institute can, with minimum effort, be reformed into several new ones, with more seats for directors and their deputies. 'Independent' international boards are then formed, from people chosen in advance.

Czechoslovakia needs the unbiased

help of international scientific organization. Candidates for study trips outside the country are chosen by competition, and it is important that there should be an age limit, and that local institutions cannot influence the results. Otherwise, older scientists can easily win stipends, presenting themselves as victims of the former regime and neglecting to say that many of them had been party members and influential bosses. The personal economic benefits are obvious. Less obvious, though, are the benefits for their home institutes. Young researchers have also become aware of the new system. We are witnessing another wave of intellectual emigration from Czechoslovakia, this time for economic reasons. This is the sixth such wave in this century, and no country can survive repeated brain-drains without serious damage.

What is even worse is 'inner emigration'. The economy is heading towards a market economy and free enterprise. International companies have quickly become established in Czechoslovakia and offer salaries to leading scientists up to ten times higher than those offered by the academy or research institutes. The maximum salary paid by the academy is about £120 a month.

It is hardly surprising that many scientists have discovered that they have a knack for entrepreneurship and start private businesses. The problem is that they often trade in the results of their ex-colleagues from research institutes. Legislation seems to be powerless or nonexistent.

To conclude, the standard of scientists in research institutes is being lowered and their average age is rising. There are some staff who had no relations with the previous regime, but many institutions still employ 'nomenclature' staff, who had accumulated a solid background under the communist regime. But not all scientists who enjoyed privileges under previous regimes were poor researchers. On the contrary, the privileges they earned through party membership (trips abroad, international congresses, material equipment, and so on) were effectively used by many of them.

It is to be hoped that the current problems of science in central Europe can be quickly remedied. Scientists need to avoid the mere imitation of Western scientific achievements and contribute to world science independently.

Zdenko Vaněk

*Division of Microbial Physiology
and Metabolites,*

*Czechoslovak Academy of Sciences,
Institute of Microbiology,
142 20 Praha 4 – Vítězná 1083,
Czechoslovakia*

Changing places

SIR — In early April, I attended a conference ("Research horizons in life sciences) organized by COPUS (Committee On the Public Understanding of Science) to facilitate interaction between scientists and the media.

One theme seemed to recur, in various guises: the difficulty of reconciling the conflicting interests of the two factions. Journalists are primarily interested in 'news' and, almost by definition, this means rapidly changing events. In a scientific context, this implies something that happened or was discovered today, not yesterday or last week, in other words a 'breakthrough'.

A frequent complaint of scientists is that the bulk of research does not proceed in this fashion. The lay public has been conditioned, particularly by television, to believe that 'breakthroughs' are the everyday currency of science. This is the exception, rather than the rule. Research is, more commonly, an evolutionary process. Edison once pithily debunked a similar myth by saying that genius was "1 per cent inspiration and 99 per cent perspiration".

A real problem of this kind of gradual development is that it is not readily newsworthy. Attempts to mould science to a news format reflect a lack of insight into scientific methods and the research process that can lead to distorted, inaccurate or sensationalist reporting of a scientist's work. Furthermore, by focusing solely on 'newsworthy' research, one is presented with a largely inaccurate image of science and scientists.

COPUS recognizes in its policy document that it must change scientists' perception of the need for a greater public understanding of science. A useful contribution to this end has been its media fellowship scheme in which academics are seconded to newspapers or television for several weeks to gain an awareness of how science is currently presented to the public.

Implicit in the media fellowship program is the premise that scientists should learn about journalism. As a scientist, I should like to suggest a reciprocal scheme whereby journalists are invited to spend, say, two weeks in a suitable scientific laboratory. More journalists might then appreciate not only how scientists think and interact but the nature of the research process. The presentation of the human face of scientists might help rid us of the tabloid 'boffin' tag. I venture that the 'diary' of such an exercise, far from being a dreary catalogue of routine, would be of general interest and, dare I say it, publishable.

Jonathan Stamford

*London Hospital Medical College,
Whitechapel, London E1 1BB, UK*

Coley's toxins in perspective

Charlie O. Starnes

Despite initial hopes, the efficacy of tumour necrosis factor in treating cancer patients has been disappointing. But a more careful selection of patients, and more appropriate treatment, might be fruitful.

EVERY year, about 3,000 people in the United States who have soft-tissue sarcomas die. The history of tumour necrosis factor (TNF) shows that one-tenth of these patients could have been saved by treatment with a bacterial vaccine. Although certainly considerably less than perfection, such odds would provide hope for an otherwise inoperable patient. In view of recent, predominantly disappointing results achieved with conventional therapies and biological products such as recombinant cytokines, a new perspective on TNF is warranted.

The history of our experience with TNF and cancer begins with the observation that certain cancer patients who developed concurrent bacterial infections would also experience concomitant remissions of their malignant disease. Recognition of this phenomenon dates back into the 1700s, about 100 years before the New York surgeon William B. Coley engineered a large-scale effort to take advantage of this observation to the benefit of his patients. Coley's interest in the subject stemmed from the loss of his first cancer patient, a young girl with a sarcoma in her right arm. Despite Coley's radical surgery, the girl developed metastatic disease and died shortly thereafter. Coley investigated the medical records of the hospital and found a seven-year-old record of a patient with a four-times recurrent inoperable sarcoma of the neck, but who was stated to have experienced a regression of the disease under the influence of an erysipelas infection (a superficial, streptococcal infection of the skin). Coley located this patient, and found him free from any clinical evidence of malignancy. He then found a substantial number of publications containing similar observations, the most frequent combination of infectious disease and cancer being erysipelas in sarcoma patients.

As a result of this encounter, Coley began the deliberate induction of erysipelas in his cancer patients, with the obvious intent of bringing their malignant disease under control. In an era before the availability of antibiotics, the problems associated with this approach soon became obvious: erysipelas was not easy to control once it began and, perhaps surprisingly, it was not all that easy to induce in the first place, some patients requiring repeated injections and others never developing the disease.

Although some dramatically definitive results were achieved, the difficulties that Coley encountered led him to the use of heat-killed versions of the streptococcus, beginning in 1892. The use of these products gave only minor therapeutic effects, and Coley decided to incorporate *Bacillus prodigiosus* (now called *Serratia marcescens*) into his vaccine, with the original idea of enhancing the virulence of the streptococcus, the two organisms being grown together in the same flask. Although this line of reasoning was later shown to be



Coley — pioneer of vaccine therapy.

erroneous (the organisms were later grown separately and then admixed), it was apparently an important step towards achieving reproducible therapeutic effects in the clinic. This combination of Gram positive, heat-killed streptococcus plus Gram negative, heat-killed *S. marcescens* is commonly referred to as "Coley's toxins", and gave Coley and his contemporaries most of their clinical successes¹.

Coley's therapy was not easy for the patient. Within an hour after injection, the patient experienced a shaking chill lasting from 10–15 minutes, followed by development of a fever in the range of 102–105 °F. Although these symptoms would subside in 12–24 hours, patients were given the injections daily or every other day for weeks to months, gradually tapering off depending on how well treatment was tolerated, and the response of the malignant disease. Despite the adverse effects, an appropriately selected patient had a reasonable chance of long-term survival (see table).

The table shows that patients with soft-tissue sarcomas are represented far more frequently (about 50%) than they occur within the human cancer population as a whole (by today's standards, only about 0.6%), and that this population of patients gave most of the dramatic responses, or 'cures' tabulated in column E. It is explained throughout the older literature that such patients were deliberately selected, as it became obvious to Coley and his contemporaries that patients with this form of the disease responded far more frequently and dramatically to the therapy than did other (unfortunately more common) forms of cancer. As Coley himself stated, "During the first few years, I treated a considerable number of cases of inoperable carcinoma . . . While in most cases a certain amount of improvement was apparent . . . in the great majority it was . . . only temporary and I decided to restrict the method chiefly to inoperable sarcoma"², and later on, in reference to his clinical results achieved by 1909, ". . . if by the administration of certain bacterial toxins we can cause the degeneration, death, and absorption of living tumor cells of one variety of cancer — sarcoma — it is not unreasonable to suppose that by the use of some other forms of bacterial toxins we may succeed in destroying or inhibiting the growth of the other and more common variety — carcinoma"¹. One of Coley's contemporaries, the Harvard physician T. W. Harmer, independently stated that the therapy "should be instituted in no case unless proven microscopically to be sarcoma"³. Coley himself reached this decision first, in 1897, and nearly 100 years later we have come to make that decision again.

After Coley's death in 1936, clinical interest in the use of his vaccine diminished in preference to the more broadly applicable chemotherapy and radiation, and it is not difficult to understand why. With these modalities it was no longer necessary deliberately to select a small subpopulation of cancer patients. Years later, when it became evident that long-term control of the disease was not being achieved, there was a renewed interest in clinical use of the vaccine⁴, which has rightly persisted⁵. Unfortunately, the necessity of selecting patients was no longer appreciated: less than 10% of the patients treated in recent times have

SUMMARY OF PATIENTS TREATED WITH COLEY'S TOXINS BEFORE 1940

Type of cancer	Total	A	B	C	D	E
Soft-tissue sarcomas	104	38	12	17	15	22
Lymphosarcomas (lymphomas)	50	24	7	4	7	8
Osteosarcoma	3	2	1	0	0	0
Ovarian carcinoma	4	1	2	0	0	1
Cervical carcinoma	2	0	1	0	0	1
Testicular	18	10	2	3	2	1
Renal	6	3	0	1	1	1
Multiple myeloma	1	0	0	1	0	0
Colorectal carcinoma	2	1	1	0	0	0
Breast carcinoma	14	8	4	2	0	0
Melanoma	6	2	3	0	1	0

Evaluation restricted to those patients considered inoperable at the time of treatment, and who had received no therapy other than the vaccine. Individual patient records are as follows: A, those making no beneficial response to treatment; B, those making an initial response but either known to relapse or lost to follow-up with 5 years; C, those rendered free of disease but lost to follow-up between 5 and 10 years; D, those rendered free of disease but lost to follow-up between 10 and 20 years; E, those rendered free of clinical evidence of disease for at least 20 years. (Source of figures is a series of articles by H. C. Nauts and G. A. Flower in the *Cancer Research Institute Monograph* between 1969 and 1975.)

been those with soft-tissue sarcomas. In addition to the lack of appropriate selection, most of the patients in these studies had received chemotherapy, radiation or both, some even receiving chemotherapy in combination with the vaccine. More recent evidence suggests that patients undergoing chemotherapy can sustain lasting alterations in various immunological parameters which can persist for 18 months after the therapy ends⁶, and may well be permanent. Given the immunological dependence of endotoxin therapy that has been described in various animal models⁷, it may have been to Coley's advantage not to have had such confounding elements to contend with.

Such problems may also be contributing factors to the recent lack of success in the clinical use of recombinant TNF and related cytokines, as most patients entering such trials have previously failed standard therapy and, for the most part, no deliberate attempt has been made to select sarcomas. The lone exception has been a study by Demetri *et al.*⁸, who initially treated a series of 36 pathologically unselected patients with a combination of TNF and interferon- γ . Because the only therapeutic responses were observed in patients with sarcoma, Demetri *et al.* deliberately selected such patients for further study; in a subsequent phase II trial, two additional responses were observed⁹. Although this decision bears obvious analogy to that made by Coley in 1897, the degree of responses observed by Demetri *et al.* was of far less magnitude than that achieved by Coley. The most likely explanation for the difference is the complexity of the patient's immune response to continuous and aggressive administration of bacterial vaccine, which would undoubtedly elicit a cascade of cytokines.

Why sarcomas should preferentially respond to Coley's treatment is an important and unresolved issue. Most sarcomas are derived from mesenchymal

tissue which is located anatomically within the mesodermal germ layer of a developing embryo. The term carcinoma does not imply embryonic derivation, rather that a malignancy has arisen from an epithelium, which in turn can originate from either endodermal, ectodermal or mesodermal germ-cell layers. In retrospect, it may be that the mesodermal embryonic origin of the tumour is the more important prognostic factor, to serve as a common denominator between the soft-tissue sarcomas and lymphosarcomas or lymphomas, which although classified pathologically as separate and distinct diseases, responded to Coley's treatment in a similar fashion; together they accounted for about 90% of the permanent responses. Further, most of the relatively few known permanent responses observed in patients with carcinoma were seen in tumours arising in tissues of mesodermal origin (see table). Coley was not the attending physician in these cases, but such responses prompted him to state in 1936, "... the number of cases of inoperable carcinoma apparently cured by the toxin treatment administered by other surgeons led me to the belief that I had greatly underestimated the value of the toxins in these cases"².

Indeed, if the mesodermal embryonic origin of the tumour does turn out to be an important prognostic factor for the prediction of responses to this form of therapy, then the population of treatable patients could well be substantially larger than Coley realized, including those with renal, ovarian and other mesodermally derived carcinomas. More recent experience with the vaccine in pathologically unselected patient populations may tend to substantiate this hypothesis; at least one dramatic response has been witnessed in a 38-year-old woman with ovarian carcinoma⁴.

The comparative sensitivity of tumours of mesodermal origin to TNF-

related therapy has been a recurrent theme ever since the initial observations of erysipelas-induced regressions in patients with sarcomas. After Coley's death, the animal models used most often in the study of this subject have been predominantly sarcomas: Carswell *et al.*¹⁰ used a sarcoma in the initial discovery of TNF; Berendt *et al.*⁷ used sarcomas to describe the essential importance of tumour immunogenicity and a corresponding T-cell immune response to the curative effects of endotoxin therapy; McIntosh *et al.*¹¹ have used a different series of sarcomas to extend these observations to combination therapy with TNF, interleukin-2 and interferon- α . It is consistent with the medical records of Coley's patients, as well as current observations in animal models, to postulate that sarcomas and other malignancies of mesodermal origin may have a higher probability of being immunogenic.

The obvious implications are twofold. Most important, serious consideration should be given to a return to an aggressive use of the vaccine in the appropriately selected patients, the optimal profile being a patient with inoperable soft-tissue sarcoma or lymphoma and no prior therapy; treatable patients may also include those with ovarian and other mesodermally derived carcinomas. Better patient selection would also undoubtedly be provided by the development of methods and procedures for establishing tumour immunogenicity in humans. Second, laboratory efforts should be concentrated on a search for the identification of a factor or factors made in response to Coley's vaccine which would have more therapeutic relevance than those that we currently possess, but perhaps to be used in combination with them. In the light of the predominantly disappointing results with chemotherapy in the treatment of the advanced stages of cancer, such an approach is certainly a reasonable place to concentrate our efforts. □

Charlie O. Starnes is at Amgen, Inc., 1840 Dehavillard Drive, Thousand Oaks, California 91320-1789, USA.

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Ever-longer sequences in prospect

The complete nucleotide sequence of a yeast chromosome is not only a landmark in the sequencing of entire genomes, but also a pointer to the benefits these projects will yield.

THE nucleotide sequence of chromosome 3 of the yeast *Saccharomyces cerevisiae* does not, as might be expected, appear with the article on page 38 describing how 35 laboratories in 17 countries have collaborated in its determination: there is simply too much of it. Although this is one of the smallest of the 16 chromosomes of bakers' yeast, some 45 pages of *Nature* would have been required to accommodate a simple representation (by the letters A, T, C and G) of the 315,000 or so bases in the entire chromosome. Moreover, there is little that could have been learned from the orthodox publication of these original data, important though they are. They can be stored only electronically and, likewise, searched only by machine.

That is one lesson to be learned from this venture, whose outcome is important in itself, but which is also another important landmark in the evolution of sequencing projects. In March, it was the 121,000 or so bases from three contiguous stretches of the genome of the nematode *Caenorhabditis elegans* (Sulston, J. *et al.* *Nature* **356**, 37–41; 1992). This month, a unique European collaboration describes a nucleotide sequence three times as long which is also, for the first time, the sequence of an entire chromosome. But these are only harbingers of a flood. The other chromosomes of bakers' yeast will punctuate the next four years, by which time the nucleotide sequence of *C. elegans* should be complete, as should be the circular genome of *Escherichia coli*. And these, of course, are only preliminaries for the various human genome projects.

That is why many readers of these documents will be concerned not only with their content but with what they promise for the future. Three questions arise, one of which is signalled by the number (147) of the authors of the article on the yeast sequence. Most of the sequencing has actually been done 'by hand', using the resources already available at the 35 collaborating laboratories, although automatic sequencing machines will no doubt be more in evidence when the project moves on to the other chromosomes. (The appearance of this article may even help to unlock the necessary equipment grants.)

The division of labour between so many laboratories is, in the circumstances, sheer necessity, given the length of the chromosome sequence, but also has several qualitative benefits. Most practically, these projects evidently need a great diversity of skills.

Sequencing technique may be the bread and butter, but so increasingly is computer skill. There may be off-the-shelf programs for compiling and interrogating databases, but a feel for their advantages and limitations somewhere in the team is crucial.

Then readers of the yeast article, as of the March article on *C. elegans*, are certain to be impressed at the degree to which sequencing strategy must be changed to suit the circumstances, perhaps an awkward cluster of repeating elements or a region in which ambiguities must be resolved by cloning a specially chosen segment of the genome and sequencing that. To what extent will it be possible to substitute artificial intelligence (in the sense of AI) for these intellectual functions? Only time will tell.

There seem also to be psychological benefits in collaborations on sequencing projects. One member of the *C. elegans* team, which is a collaboration between the Medical Research Council Laboratory of Molecular Biology at Cambridge, England, and Washington University at St Louis, Missouri, calls the phenomenon "hybrid vigour". The yeast project, supported by the European Commission's Biotechnology Division, has by contrast brought together a great diversity of laboratories, not all of them equally renowned; the result seems to have been that the less well-known laboratories have been challenged to function well, while others have learned unfamiliar managerial skills.

So what do the results so far suggest will flow from the larger sequencing projects now, or soon to be, under way? The most obvious surprise, with the yeast chromosome and *C. elegans*, is that there are more genes in the regions sequenced than had been expected. The nematode, for example, is now expected eventually to yield 15,000 genes (an estimate confirmed by the same collaboration's cDNA analysis in the May issue of *Nature Genetics* (Waterson, R *et al.* **1**, 114–123, 1992)).

Chromosome 3 of yeast is also replete with previously unknown genes, or at least with open reading frames from which mRNA molecules can be transcribed when there are appropriately placed regulatory elements to turn them on. Indeed, the genetic map of the chromosome contained only 34 genes before the complete sequence was assembled, but now there are 182, not counting tRNA genes, transposable elements and genes with fewer than 300 bases (of which there appear to be only four). Allowing for a small hand-

ful of genes previously sequenced but not mapped, the result is that chromosome 3 has 145 new genes, or that its gene content is five times greater than was previously believed.

There are also, of course, the familiar oddities of improbable similarities with genes first recognized in other organisms — the *nifS* gene of nitrogen-fixing bacteria, for example. The authors themselves ask why there should be the equivalent of an essential component of the mechanism for fixing nitrogen in an organism such as yeast that does not fix nitrogen. The answer says much about the way in which chance homologies may fortuitously be invested with an air of magic. If, as is now suggested, the function of the protein product of this *nif* gene is tied up with mitochondrial metabolism, it is not all that surprising that both yeast and bacterial cells should use something very similar. The interesting question is simply that of how two such different organisms acquired such similar genes.

That question points to the third issue raised by the appearance of these articles (and the flood that will soon follow): is there not a quicker way to the listing of the genes in an entire organism by means of cDNA analysis? Dr Craig Venter at the US National Institutes of Health (NIH) has recently made most of the running in this field, which has been further dramatized by the decision of the NIH to seek patent protection for his gene fragments (which are not DNA complements of entire protein products, but simply long 'tags' corresponding to the end of them).

The truth, which the *C. elegans* and yeast studies make perfectly plain, is that cDNA analysis and the sequencing of entire genomes are not alternatives, but are rather complementary approaches. By itself, cDNA analysis can only assist with the enumeration of genes, but cannot by definition throw much light on regulatory processes, on the reasons why some genes have introns and others do not, on the intriguing tendency for tRNA genes and transposable elements to be juxtaposed and on questions such as how the genome got like that, anyway. On the other hand, cDNA analysis should prove to be a useful means of refining the definition of the probes and primers on which the sequencers have to rely. In the wake of James Watson's departure from the US Human Genome Project, there is a danger that the cDNA approach will be offered as a cheaper alternative to complete sequencing, which it is not.

John Maddox

Hidden lymphocytes

Anthony L. DeFranco

THE prevailing view of autoimmune diseases is that they represent failures of immunological tolerance to self-antigens. On page 77 of this issue¹, a group at Kyoto University (Murakami *et al.*) report observations with anti-erythrocyte immunoglobulin-expressing transgenic mice demonstrating that autoantibody production can result from atypical B lymphocytes that evade self-antigen-induced elimination. These experiments illustrate the importance of tolerance induction in the B-lymphocyte compartment, and point to a mechanism by which autoimmunity might arise.

Transgenes

Transgenic mice have provided a powerful window into the underlying basis of immunological tolerance^{2,3}. New protein antigens can readily be expressed from transgenes, and their effects on antigen-specific lymphocytes assessed. These neo-self-antigens are typically expressed continuously, and so are much more akin to true self-antigens than are injected antigens. Perhaps even more powerful is the approach of introducing functionally rearranged genes encoding lymphocyte antigen receptors (membrane immunoglobulin or T-cell antigen receptor) into the mouse germ line to create mice with large numbers of B cells or T cells of identical specificity. This technique makes it possible to follow the fate of antigen-specific lymphocytes that

encounter antigen in various contexts.

The Kyoto group used genes encoding an anti-erythrocyte antibody to create transgenic mice with B cells that were almost all specific for an erythrocyte self-antigen⁴. About half of the mice exhibited an autoimmune haemolytic anaemia, indicating that immunological tolerance was only partially able to protect the animals from attack by the self-reactive B cells. The mice did exhibit a dramatic reduction in the numbers of B cells in spleen and lymph node; presumably, tolerance mechanisms caused death of most of the self-reactive B cells. In the peritoneum, very few B cells were present soon after birth, but the numbers increased with age until by adulthood roughly normal numbers had been attained. In addition to conventional B cells, the peritoneum is rich in a second type of B cell called the Ly-1⁺ B cell or, more recently, the B-1 cell⁵. B-1 cells arise early in development and in adoptive transfer experiments have the potential to renew themselves^{6,7}. They may represent a lineage separate from the conventional B cells of spleen and lymph node (see box).

An attractive hypothesis is that a few B-1 cells are somehow spared elimination at the hands of the self-antigen, and that they migrate to the peritoneum, where, in the absence of the self-antigen, they can proliferate until normal numbers are achieved. To test this hypoth-

esis, Murakami *et al.*¹ injected red blood cells into the peritoneal cavity of their transgenic mice. The results were dramatic — within 12 hours almost all of the transgenic B-1 cells had died. The presence of fragmented DNA suggests that these cells had undergone programmed cell death by apoptosis. Thus, contact with the self-antigen induced rapid death of those cells that had previously avoided inactivation. They were not inherently resistant to immunological tolerance mechanisms such as clonal deletion, but rather were resident in a privileged environment in which the self-antigen was absent.

Some of the B-1 cells from the peritoneum of the transgenic mice differentiated into cells secreting antibody at a high rate. The number of these autoantibody-secreting cells in the peritoneum correlated with the severity of the haemolytic anaemia, and such cells were not found elsewhere¹. These autoantibody-secreting cells disappeared following weekly injection of red blood cells into the peritoneal cavity, and the autoimmune haemolytic anaemia rapidly cleared up. Thus, the conclusion that B-1 cells in the peritoneum are responsible for autoantibody production seems likely to be correct.

Autoantibodies

Although the involvement of B-1 cells in autoimmune diseases has not been firmly established, it has been shown that these cells from non-transgenic mice secrete a number of autoantibodies and that in certain autoimmune diseases of man and mouse⁶ their numbers are increased. The

How do B-1 cells originate?

THE CD5⁺ or B-1 lymphocytes⁵ exhibit a distinctive cell-surface phenotype, a characteristic homing to the peritoneal and pleural cavities, and an unusual capacity for self-renewal^{6,7}. A number of self-reactive antibody specificities are greatly enriched in this B-cell subpopulation.

Two models have been proposed to explain the origin of B-1 cells. First, they could derive from developing B-lineage precursors that are committed to become B-1 cells. According to this hypothesis, B-1 cells and conventional B cells represent separate cell lineages, as is believed to be the case for T cells expressing either $\alpha\beta$ T-cell receptors or $\gamma\delta$ T-cell receptors¹¹. This view has received strong support from adoptive transfer experiments, which showed that adult bone-marrow progenitors repopulate the B-1 cell population only poorly, whereas fetal liver or omentum progenitors do so quite effectively^{12,13}. The

lymphoid progenitors in fetal omentum, unlike those in fetal liver or adult bone marrow, only reconstituted B-1 cells and did not reconstitute conventional B cells in SCID mice¹³. Moreover, cotransfer of immunoglobulin allotype-marked adult bone-marrow stem cells and fetal liver stem cells resulted in B-1 cell production from the fetal liver precursors but not from the adult bone marrow precursors, demonstrating that the ability to generate B-1 cells is an intrinsic property of the fetal B-cell precursors¹².

Although a great deal of evidence favours the separate-lineage hypothesis, it is possible that B-1 cells instead represent a long-lived physiological state that results from a particular type of contact with antigen of a previously uncommitted B cell. Evidence for this view comes from experiments in which *in vitro* treatment of conventional B cells with anti-immunoglobulin antibodies and interleukin 6 leads to a cell-surface

phenotype that closely resembles that of B-1 cells¹⁴. The observation that certain immunoglobulin-transgenic mice seem predominately to express either conventional B cells¹⁵ or B-1 cells¹⁶ also seems to fit this hypothesis better, as different membrane immunoglobulins might differ in their interactions with environmental antigens.

Nonetheless, the adoptive transfer experiments cannot be readily explained by a model invoking one B-cell lineage, and more information will be required to reconcile the current data with one or other of the two hypotheses. A possible explanation is that B-1 cells could derive from committed progenitors that require contact with antigen in a particular form to enter the long-lived, self-renewing B-1 cell population. In any case, a greater knowledge of the origin and properties of B-1 cells is important for understanding normal and autoantibody immune responses.

A.L.DeF.

experiments of Murakami *et al.* demonstrate that B-1 cells can be responsible for autoantibody production, and that such antibody production can lead to autoimmune disease.

Several other groups studying tolerance with transgenic mice have also uncovered circumstances in which tolerance breaks down. For example, in one line of mice expressing simian virus 40 T antigen in the pancreatic β cells, onset of expression is delayed until adulthood and autoimmune destruction of the pancreas follows⁸. From these results it seems that expression of self-antigens may need to commence soon after birth for tolerance to become established, in agreement with the classical view of tolerance. In separate experiments, mice expressing, in the pancreatic β cells, a transgene encoding glycoprotein from the lymphocytic choriomeningitis virus exhibited tolerance to both the glycoprotein and the β cells. T-cell receptor transgenes were then introduced into these mice. The tolerance of T cells expressing these receptors appeared to be due to 'ignorance' of the antigen rather than to deletion or inactivation of the antigen-specific lymphocytes⁹. Infection of these mice with lymphocytic choriomeningitis virus, however, led to an immune response that attacked the β cells^{9,10}.

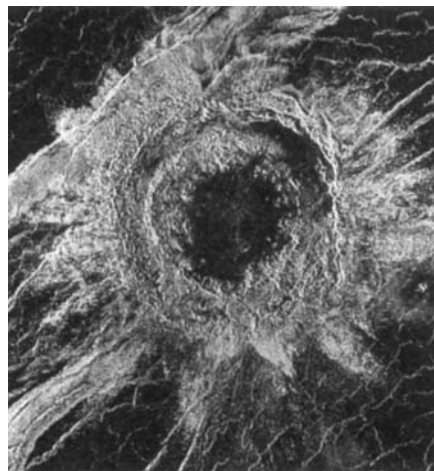
These examples, and that now provided by Murakami *et al.*, have increased our knowledge of immunological tolerance, particularly of the circumstances that can result in a failure of tolerance and the initiation of an autoimmune response. Ultimately, an understanding of how autoimmune disease commences might lead to effective strategies for its prevention or treatment. □

Anthony L. DeFranco is in the Departments of Microbiology and Immunology, and Biochemistry and Biophysics, and in the George Williams Hooper Foundation, University of California San Francisco, San Francisco, California 94143-0552, USA.

Killer acid at the K/T boundary

William B. McKinnon

To many in the field, it is becoming undeniable that the reign of the dinosaurs, along with perhaps 75 per cent of all other species, came to a close at the end of the Cretaceous period (65 million years ago) when a massive asteroid or comet struck the Earth in the Caribbean near present-day Chicxulub, Mexico¹. Confirmation was presented at the recent meeting of lunar and planetary scientists* that the geophysical anomaly at this site is due to a *bona fide* impact.



Klenova (pictured here by NASA's Magellan radar mapper) is a 145-km-diameter multiringed basin on Venus (the most Earth-like of the planets) which may have been created relatively recently by a high-velocity impact like that at Chicxulub. Perhaps the Chicxulub structure, when fresh, resembled this.

Although it is not yet precisely dated, the hidden, roughly 180-km-wide crater sits at the geographical centre of the thickest Cretaceous/Tertiary (K/T) boundary-layer deposits. Its detailed geology also suggests how the global ecosystem was punished by the impact.

Chicxulub, on the Yucatan Peninsula, was originally described as an impact structure 10 years ago², on the basis of an intense circular magnetic pattern. But only last year did it receive widespread attention and was the connection with the K/T extinctions made. Since then, numerous samples of drill cores from the site, supposedly lost, have turned up. V. L. Sharpton (Lunar and Planetary Institute), formerly sceptical that Chicxulub was an impact structure, presented results from a US-Mexican collaboration detailing petrographic evidence in these cores of a high-velocity impact: pronounced brecciation (fragmentation), glass, and shocked quartz and feldspar (minerals characteristic of the andesitic

basement in the region). The breccia contains minerals indicating a range of shock levels (equivalent to radial distances from the impact point), from pressures of less than 10 gigapascals up to 23 gigapascals. It also contains diaplectic glasses (minerals that have been amorphotized but not truly fused), which indicate more severe shocking.

In addition, there are shocked grains and rock fragments as inclusions in andesitic glass, with some of the inclusions partially digested. The glasses themselves show structures due to internal flow. In all, as Sharpton affirmed, this is conclusive geological evidence that the 180-km-diameter circular gravity and magnetic anomaly at Chicxulub was created by a high-velocity impact. The crater is now buried by younger sedimentary rocks, but when it was young, it may have looked something like Klenova crater, a recent impact structure on Venus (see figure).

Chicxulub, Mayan for "tail of the devil" (according to one of the less suggestive translations), was the site of a submerged sedimentary carbonate/evaporite platform at the end of the Cretaceous. Ahrens and O'Keefe³ had earlier pointed out the consequences of a large impact into carbonate-bearing rocks: the resulting flux of CO₂ into the atmosphere would lead to a severe greenhouse-driven temperature rise that might last several tens of thousands of years (until the CO₂ could be reabsorbed by the oceans). But oxygen isotopes of fossiliferous sediments⁴ and a palaeobotanical study of leaves⁵ (but see Scientific Correspondence in *Nature*, 26 March 1992) indicate an initial cooling at the K/T boundary, followed by a warming lasting many thousands of years.

The answer may lie in geology of the northern Yucatan, where in addition to dolomitic limestones there are also massive beds of anhydrite⁶, CaSO₄, with an integrated thickness of several hundred metres to over a kilometre. The impact vaporization of anhydrite would have released large amounts of SO₂ into the stratosphere where, as following powerful volcanic eruptions, it could ultimately have combined with water to make sulphuric acid, H₂SO₄; the impact would also have supplied its own water in the form of vaporized ocean (R. Brett, US Geological Survey, Reston; K. O. Pope, Geo Eco Arc Research; E. C. Perry, Northern Illinois University). The resulting sulphuric-acid aerosol would have been a strong scatterer of visible solar radiation, and so could have led to cooling of the stratosphere and tropo-

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sphere and darkness at the surface. These in turn could have halted photosynthesis and caused the food chain to collapse. Such cold and dark would have outlasted any caused directly by dust lofted by the impact, as dust would have settled out of the stratosphere in a few months. The beautiful red-orange Pinatubo sunsets that most of us (save some astronomers) have been enjoying is a case in point. The dust from this Philippine eruption is gone, but sulphuric-acid aerosol remains in the stratosphere, deflecting blue light and causing the evening sky to burn brilliantly.

Equally devastating to the dinosaurs and other forms of life may have been the subsequent rainout of sulphuric acid from the stratosphere. Geochemical evidence of such increased acidity takes the form of a spike in $^{87}\text{Sr}/^{86}\text{Sr}$ in seawater across the K/T boundary (reflected in fossil foraminifera). The spike is of such a magnitude that it may only have been produced by a sudden increase in the weathering of continental surface rocks⁷. The agents responsible have until now been considered to be nitric and nitrous acids, produced from nitrogen oxides created in the atmosphere by the shock waves of the K/T impactor, by its ejecta, or by global wildfires (along with a possible but smaller contribution of HCl from vaporized seawater).

The amount of nitrogen oxides produced is estimated^{8,9} to have been between 10^{14} and 10^{17} moles. The amount of sulphur directly released to the atmosphere from vaporized and decalcinated anhydrite may have been 10^{16} moles or greater (Brett; Pope; Perry). Thus the amount of sulphuric acid returning as rain, potentially 1–10 kg per square metre or more, with twice the molar acidity of nitric acid, competes in destructive power with the nitric and other acids. If it rained out quickly enough (over a few years), it would have been enough to turn the Earth's surface into one vast killing field.

A quantitative assessment of the SO_2 , CO_2 , H_2O , Cl, CaO and so on released to the atmosphere by the Chicxulub impact is still wanting. Of particular interest are the removal and rainfall times and whether the cooling due to H_2SO_4 really could have counteracted CO_2 greenhouse warming. It also re-

mains to be evaluated whether sufficient H_2SO_4 can form in the absence of sunlight (photochemistry is important for its formation in the present-day stratosphere), and whether the large amount of highly reactive CaO released could have largely sequestered SO_2 and CO_2 to reform inert CaSO_4 and CaCO_3 (B. Fegley, personal communication).

Argument continues over the boundary clay in western North America; the clay there exhibits a doublet stratigraphy interpreted alternatively as a layer of ejecta overlain by a layer of vapour plume debris (B. F. Bohor, USGS, Denver), or as ejecta from two distinct impacts (separated by enough time for plants to take root; E. M. Shoemaker, USGS, Flagstaff). Regardless, the geology of the Chicxulub region provides a

good match for the geochemistry of the tektite glasses in the 0.5-m thick K/T layer in Haiti, 900 kilometres away). These glasses have been precisely dated¹⁰ at 64.5 ± 0.1 million years old. The bulk composition of Haitian tektite glass is andesitic, but some samples have anomalously high amounts of CaO and S. An anhydrite source for these is indicated and experimentally supported^{6,10}. It would be startling if the nearby and very large Chicxulub impact is not of the right age to be the source of the Haitian K/T glass. □

William B. McKinnon is in the Department of Earth and Planetary Sciences and McDonnell Center for the Space Sciences, Washington University, Saint Louis, Missouri 63130, USA.

CYTOKINES

Poking holes in the network

William E. Paul

CYTOKINES produced by, and acting on lymphocytes and other cells of the immune system, regulate responses of that system in health and disease. But their physiological functions have been difficult to determine despite (or possibly because of) their plethora of activities *in vitro*. Over the past year or so, three cytokines, interleukin-2 (IL-2), IL-4 and IL-10, have been subjected to the ultimate test through deletion of their genes in mice by targeting techniques. The results, some expected and some surprising by their absence, were discussed at a meeting on cytokines* earlier this year.

A. Schimpl (Univ. Würzburg) summarized work on IL-2-deficient (IL-2⁻) mice produced in Ivan Horak's laboratory. Interleukin-2 has been generally regarded as a major growth factor for T cells, and the most interesting feature of these studies (a preliminary report of which has been published¹) is how little the immune system seems to be perturbed in its absence. Indeed, characterization of these mice had revealed no major defects in T-cell development within the thymus. Among young mice, peripheral T cells also showed a normal distribution. As expected, there was a diminution of *in vitro* proliferation of T cells to mitogen and of the *in vitro* generation of cytotoxic T cells, reflecting the fact that T-cell production of IL-2 is important in both these responses. But even the defect in proliferation was only partial, affirming the well-recognized redundancy of control mechanisms in the cytokine system².

More intense study now indicates that deleting the gene for IL-2 does have important, if somewhat subtle, effects on the system even in young mice. The principal disturbance is on the balance of lymphokines produced, a dysregulation that is consistent with the idea that lymphokines produced by CD4^+ T cells regulate one another's production³. Indeed, among long-term clones of CD4^+ T cells, IL-2 and interferon- γ (IFN- γ) are mainly produced by Th-1 clones whereas IL-4, IL-5, IL-6 and IL-10 are principally produced by Th-2 cells⁴. In the IL-2⁻ mice, some of the 'Th-2 type' lymphokines (IL-4, IL-6 and IL-10) were overproduced upon *in vitro* stimulation, and serum titres of IgG1 and IgE, both of which are regulated by IL-4, are strikingly increased. Indeed, IL-4 knock-outs (see below) dramatically confirmed the IL-4 effect on serum IgE and IgG1; R. Locksley (Univ. California, San Francisco), A. Sher (NIAID, Bethesda) and I emphasized the profound effect of the presence of IL-4 at the time of priming of an immune response — the consistent finding is that IL-4 enhances its own production and inhibits the production of IFN- γ , thus biasing responses towards a Th-2 phenotype.

The extent of the dysregulation in IL-2⁻ mice implies that IL-2 is also involved in establishing balance in lymphokine production in immune responses by favouring the appearance of T cells that produce IL-2 and IFN- γ . Because there is much evidence — described by Locksley, Sher, B. Bloom (Albert Einstein College of Medicine, New York) and H. Morse (NIAID) — that specific lymphokines made in re-

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* Cytokines in Normal and Abnormal Immune Regulation, Asilomar Conference Center, Pacific Grove, California, 25–28 January 1992.

sponse to particular infectious agents determine the protective value of the immune response, IL-2⁻ mice would be expected to show striking susceptibility to certain types of infectious agents.

Schimpl also reported that the relatively subtle abnormalities of young IL-2⁻ mice give way to a striking lymphadenopathy and splenomegaly in older mice, associated with an increase in immature B lymphocytes and granulocytes in the spleen. This is followed by an almost total loss of B220⁺ cells, first from the bone marrow and later from the spleen. Most animals die within 2 to 6 months of age. Detailed study of these mice, now in progress, should yield illuminating information about the normal physiological functions of IL-2 and the consequences of an unbalanced cytokine network.

W. Müller (Univ. of Cologne) described experiments on mice in which the IL-4 and IL-10 genes had been inactivated by gene targeting; these mice are referred to as IL-4T (for targeted) and IL-10T, respectively. The IL-4T mice had already been described⁵, but this was the first report of the phenotype of the IL-10T animals.

The IL-4T mice have an abnormality that is essentially predictable from studies *in vitro* (showing that IL-4 regulates IgG1 and IgE production by controlling immunoglobulin class switching to these isotypes^{6,7}) and from experiments *in vivo* (showing that antibodies to either IL-4 or its receptor completely prevent increases in serum IgE to helminthic infection or to polyclonal B cell activation, stimulants that often increase serum IgE levels 100-fold or more⁸). Not surprisingly, then, the IL-4T mice had no detectable serum IgE and markedly diminished IgG1. They failed to produce IgE in response to nematode infection and showed a strikingly diminished production of IgG1, but not of other IgG isotypes, in response to conventional thymus-dependent immunization.

These results have several implications. First, it is now abundantly clear that IL-4 is absolutely essential for production of IgE. Second, IL-4 is a physiological switch factor for IgG1, which had been in doubt, although it cannot be the only one because some IgG1 is made by the IL-4T mice. Finally, the reciprocal phenotypes of the IL-4T and the IL-2⁻ mice, in terms of the dominant types of serum immunoglobulin, emphasize the different regulatory properties of the two lymphokines, and of the T cells that produce them. They lead to the prediction that IL-4T mice will show diminished production of other Th-2 products and that they will overproduce IFN- γ .

Interleukin-10 is a more recent entrant on the cytokine scene but the range of its

activities is impressive and growing, typical of this class of molecules for which the description "pleiotropic and redundant" has been popular. A particularly provocative observation (M. Howard, DNAX, and ref.9) is that treatment of normal mice with monoclonal anti-IL-10 antibody from birth causes a striking reduction of serum IgM concentrations and the elimination of CD5⁺ B cells, which had been postulated to be the principal source of serum IgM¹⁰. These effects seem to result from the action of IL-10 as a "cytokine synthesis inhibitory factor" (the activity under which it was initially described)³. Indeed, anti-IL-10 treatment causes the overproduction of IFN- γ and anti-IFN- γ reverses the effects of anti-IL-10. Somewhat disappointingly, the IL-10T mice had normal serum IgM levels and showed no evidence of the overproduction of IFN- γ . These observations imply that the effects obtained by *in vivo* neutralization of IL-10 with antibody may have a more complex explanation, or that IL-10T mice may have activated mechanisms to correct the abnormalities that would normally result from the absence of the cytokine.

The IL-10T mice tended to be smaller than their IL-10 'sufficient' littermates, and some of them died early in life. Although the reasons are not yet known, detailed study of these valuable animals will offer much information regarding the contribution of IL-10 to the cytokine network.

Studies of the consequences of deleting key players in the regulation of the immune system provide a striking glimpse of how the immune system controls itself in the course of immune responses. Further analyses of the resistance of these mice to various infections will provide much insight into the contribution of different cytokines to protective responses and into the complex interplay between these potent molecules. □

William E. Paul is in the Laboratory of Immunology, NIH National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892, USA.

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RÉSUMÉ

Defence costs

A fresh twist in the evolutionary struggle between brood parasites and their hosts is described in a study of the yellow-browed leaf warbler, a presumed former host of cuckoos. Sharp-eyed leaf-warbler females strongly discriminate against eggs of other species, turning them out of the nest. But K. Marchetti (*Proc. R. Soc. B* **248**, 41–45; 1992) has found that the birds are sometimes too picky for their own good and reject their own eggs. This cost of egg discrimination could lead to the selective loss of host defences, which in turn would make the leaf-warbler population vulnerable to re-exploitation by cuckoos. That, says Marchetti, might explain the survival of brood parasitism despite its burden on the host species.

Tickled by light

FISH without contractile pupils adjust to light and dark by changing the lengths of their visual receptors. The rods, which sense dim light, shorten on dark adaptation and the cones lengthen. This allows weak incident light to get at the rod outer segments first, whereas in bright light they are shielded by the retinal pigment layer. Now K. Pagh-Roehl *et al. (Cell Motil. Cytoskel.* **21**, 235–251; 1992) have found that elongation of the rods can be reproduced in a dark-adapted preparation *in vitro*. The contractile element of the rod inner segment contains actin filaments, which push outwards by elongating at their slow-growing ('pointed' ends), while depolymerizing at the barbed ends. How they are induced to do this is not clear.

Superfluid solution

THE next assault on the solar-neutrino problem may use a new kind of neutrino detector devised and tested by S. R. Bandler *et al. (Phys. Rev. Lett.* **68**, 2429–2432; 1992). The snag has been that most detectors are sensitive only to high-energy neutrinos, so neglecting the contribution from hydrogen-burning nuclear reactions in the Sun's core. Gallium detectors go but part way to resolving the difficulty. Bandler *et al.* note that a neutrino passing through superfluid helium can create one of the exotic menagerie of collective quantum excitations, such as rotons, possible in this material, which persist until they reach the superfluid's surface. Here they dissipate their energy by evaporating a few atoms which can condense on a nearby ultracold wafer. The resulting thermal pulse of the wafer signals the neutrino's arrival. The detector's main advantage, besides the low threshold energy, is that the superfluid bath can be made as large as necessary to maximize the number of scattering events without compromising sensitivity, which depends on the wafer's mass.

Middle atmosphere cooling

Rolando R. Garcia

CHANGES in a thin layer of sodium vapour, about 90 km above the Earth's surface, could be revealing the far-reaching effects of greenhouse gases, B. Clemesha, D. Simonich and P. Batista suggest in *Geophysical Research Letters*¹. The authors have been monitoring the layer since 1972, and find that by 1987 its mean height had fallen by nearly a kilometre. They attribute the drop to greenhouse gases, which besides warming the lower atmosphere, can actually cool the middle atmosphere.

Although the mesospheric sodium layer was discovered more than 60 years ago, most geophysicists have tended to regard it as a curiosity, if indeed they were aware of its existence. It is believed to be produced by ablation of meteorites as they enter the atmosphere. The amount of sodium in question is very small — maximum concentrations reach only 3–4 thousand atoms per cm³ near an altitude of 90 km, or less than 0.1 parts per billion by volume.

Nevertheless, the layer is easily detected from the ground because of the strong emission by the orange sodium doublet (at a wavelength of 589–589.6 nm). It is now known that the sodium layer is sharply peaked near a height of 90 km, with a width at half-maximum of about 10 km. Theoretical studies and numerical models suggest that the sharpness of the layer is due to removal of atomic sodium below 90 km by the reaction $\text{Na} + \text{O}_2 + \text{M} \rightarrow \text{NaO}_2 + \text{M}$, where M is any inert molecule. Above 90 km, photoionization and charge exchange processes produce Na^+ , which is thought to be removed ultimately by incorporation into hydrates.

Lidar ranging

Clemesha and his colleagues have been making lidar (laser-infrared ranging) measurements of the mesospheric sodium layer from São José dos Campos, Brazil, since 1972. In their new paper, they report that the height of the centroid of the sodium distribution has decreased by 49 ± 12 m a year, or a little over 700 m over the period 1972–87. To connect this finding with global cooling of the middle atmosphere, the authors note that a uniform cooling of 5 K at all altitudes above 50 km would be sufficient to account, through contraction of the atmosphere, for a lowering of about 700 m of constant-density surfaces in the vicinity of 90 km. The altitude at which meteorites ablate and deposit sodium depends on atmospheric density, so a lowering of the density surfaces would cause a corresponding lowering of the

source of sodium and, presumably, a decrease is the mean altitude of the sodium layer. Clemesha and colleagues suggest that the required temperature decrease is consistent with some estimates of temperature trends in the stratosphere and lower mesosphere².

Global cooling of the middle atmosphere due to increasing concentrations of greenhouse gases has been predicted by several investigators from the results of numerical models. In a recent paper, Roble and Dickinson³ showed that the same gases (CO_2 , nitrous oxide, methane, fluorocarbons) that produce the greenhouse effect in the troposphere also cool the upper layers of the atmosphere by increasing the amount of infrared radiation emitted to space. The work of Brasseur and Hitchman⁴ leads to similar conclusions for the entire layer between heights of 25 and 70 km. Whereas Roble and Dickinson studied the response of the upper mesosphere and thermosphere, the results of Brasseur and Hitchman are, if anything, more relevant to the hypothesis put forward by Clemesha and colleagues because they demonstrate that global cooling should occur throughout most of the stratosphere and mesosphere.

For a doubling of atmospheric CO_2 (and corresponding increases in the concentrations of other radiatively important trace gases), the numerical models predict temperature decreases in the middle atmosphere ranging from 2–3 K at 25 km to more than 20 K near 45 km. Above this altitude, the calculated cooling decreases to a minimum in the upper mesosphere before increasing sharply again in the thermosphere above 90 km. However, these predictions are based upon estimates of the expected concentration of greenhouse gases at the end of the next century. Cooling of the middle atmosphere resulting from the increases in greenhouse gases that have taken place over the past 15–20 years should be substantially smaller — a maximum of 2–2.5 K near 45 km and proportionally lesser amounts elsewhere. These figures are in broad agreement with analyses of temperature trends performed by the International Ozone Trends Panel⁵, which indicate that temperatures decreased by about 2 K in the upper stratosphere and lower mesosphere during the 1980s.

Although models and observations both lend qualitative support to Clemesha and colleagues' suggestion, the best estimates of temperature decreases in the middle atmosphere appear to be too small to produce the effect

claimed by these authors. In particular, the maximum temperature decrease at any altitude in the middle atmosphere during the period 1972–87 should be about 2 K, whereas Clemesha and colleagues require a uniform change of 5 K between 50 and 90 km. Even if one assumes that, as predicted by the numerical models, temperature decreases occur everywhere between 25 km and 90 km, and that their average magnitude over this range of altitudes is as large as 1 K, constant density surfaces in the vicinity of 90 km would be lowered by only 250 m, just a third of the change reported for the sodium layer.

Removal processes

But Clemesha and colleagues do not consider the influence of removal mechanisms on the vertical distribution of sodium. The input of meteoric sodium⁶ is rather sharply peaked at about 82 km, but the layer itself is centred at 90 km. This implies that the altitude of the layer depends strongly on the vertical distribution of removal processes and not just on the altitude at which meteors ablate. The rapid decrease of sodium below the peak at 90 km is believed to be due to removal by its three-body reaction with oxygen, whose efficiency varies as the square of atmospheric density. When this dependence is taken into account, a simple calculation assuming a 1 K temperature drop between 25 and 90 km yields a decrease of over 500 m in the altitude of the sodium peak, not far off the 700 m reported by Clemesha and colleagues.

Although it is not possible to state with absolute certainty that the decrease in the altitude of the sodium layer is an indication of global cooling in the middle atmosphere, the behaviour of the layer clearly bears systematic watching in years to come. Like the apparent increase in the frequency of polar mesospheric clouds reported recently in this journal by Thomas *et al.*⁷ and attributed to increases in mesospheric water vapour, it may be that the initial signs of global change are to be sought in the upper reaches of the atmosphere. □

Rolando R. Garcia is in the Atmospheric Chemistry Division, National Center for Atmospheric Research, PO Box 3000, Boulder, Colorado 80307, USA.

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Time travel on a string

Bruce Allen and Jonathan Simon

TIME machines are the stuff of good science fiction and bad Hollywood movies — one would think. But about a year ago, J. R. Gott proposed¹ a simple way to build such a machine out of two long straight pieces of the exotic form of matter called cosmic strings. Whether cosmic strings really exist is not known, but that does not diminish the elegance of Gott's design, which does not seem to violate any known laws of physics. The enthusiastic debate over whether such a machine might indeed be built and function as advertised has stimulated several highly respected research groups to examine the relevant fundamental principles in much greater detail²⁻⁵. Indeed, Hawking has formulated a "Chronology Protection Conjecture"⁵ which, if correct, would rule out time machines altogether. In fact, the parts that have

already been proved suggest that attempts to build a time machine from Gott's idea are doomed.

The notion of a time machine is simple enough. Fortifying yourself with a freshly brewed cup of coffee, you step into it at 9 AM. A few seconds later (according to your wristwatch) you step out again, finding yourself surrounded by the aroma of coffee brewing, with the clock on the wall reading 8.30 AM, that same morning. Seating yourself comfortably in the corner, you can now watch yourself drink your coffee and step into the time machine at precisely 9 AM.

The enigmatic nature of this situation is familiar to even the most casual reader of science fiction. Suppose the time traveller knocks over the coffeepot at 8.45 AM, or disables the time machine, or even murders the other copy of himself? Then the time traveller could prevent himself from making the voyage: a clear contradiction or paradox. For this reason, it has generally been thought that time travel is impossible. But work by Friedman, Thorne and others⁶⁻⁸ has established the likelihood that a "consistent" solution is possible in general — in this case, one for which the time traveller, stepping into his machine, sees another copy of himself sitting quietly in the corner chair, watching. As long as there is at least one consistent solution, the question of free will may be quietly ducked, just as it is for the ordinary, deterministic physics of Newton.

The question "can one build a time machine?" can be posed precisely in the language of Einstein's theory of general relativity. This theory describes space (x, y, z) and time (t) as a unified four-dimensional continuum (t, x, y, z) distorted and bent by the gravitational field of matter. Any object follows a curve through this spacetime; for example a person sitting in a chair at the space point $x=y=z=0$ follows a 'timelike' curve described by the line consisting of the points (all $t, x=y=z=0$). Viewed this way, the time-traveller follows a curve through spacetime which starts at some initial spacetime point (t, x, y, z), moves forward in time (as measured by the traveller's watch) but which nevertheless returns to the same spacetime point (t, x, y, z) along a 'closed timelike curve'. Because it is matter that determines the way that spacetime curves, the crucial question is: can one generate closed timelike curves using only the different types of matter that exist?

Four years ago, Morris and Thorne^{9,10} designed a time machine based on the Casimir effect. The Casimir effect pro-

duces a region in which the energy of all the matter contained is negative (and so also the mass, according to Einstein's famous equation $E = mc^2$). Roughly speaking, this is possible because even empty space has free energy due to quantum fluctuations. With electrically conducting boundaries (metal plates), it is possible to suppress some of these fluctuations, reducing the energy below the zero-point level. Time machines of this type, which rely on negative energy sources, are not as troubling as Gott's time machine, because known physical laws might well conspire to keep negative energy from building up to the degree necessary to allow closed timelike curves.

Gott's design, which relies on cosmic strings, does not suffer this way. In 1976, Kibble discovered¹¹ that such matter is predicted by 'gauge' theories, the best existing mathematical description of the behaviour of matter at very high energies and densities. Astronomers have so far failed to find any cosmic strings, but nevertheless they may have been significant in allowing large-scale cosmological structures like galaxies to develop.

Although their diameter would be only 10^{-29} cm, cosmic strings would typically pack 10^{22} g into each centimetre of their length. In the early 1980s, Gott¹² and Hiscock¹³ independently discovered that cosmic strings would bend and distort spacetime in a very peculiar way. The spacetime outside the string is uncurved, or flat (there are no gravitational forces), as if no string were present. But the circumference of a circle centred on the string is less than the euclidean circumference, $2\pi r$, as if a wedge of angle α had been cut out of a circular sheet of paper, and the edges glued together to form a cone (Fig. 1). The angle α , the deficit angle, would be about $1/10,000$ of a degree for a realistic cosmic string (in our figures we take α to be 90° to exaggerate the effects). While the flat space outside the string is locally no different from what it would be in the absence of the string, its global properties are affected.

For example, two initially parallel light rays that pass by the string on opposite sides become focused together and eventually cross paths (Fig. 1, bottom). Gott's time machine, constructed with two infinitely long, straight cosmic strings, moving at high velocity (Fig. 2), exploits this ability of strings to bend spacetime and to focus light rays. To see how it works, one need only understand first how objects (or observers) behave in a spacetime with a cosmic string, and second how Einstein's theory of special relativity changes the way time is measured by observers moving at different speeds.

As described above, the space around

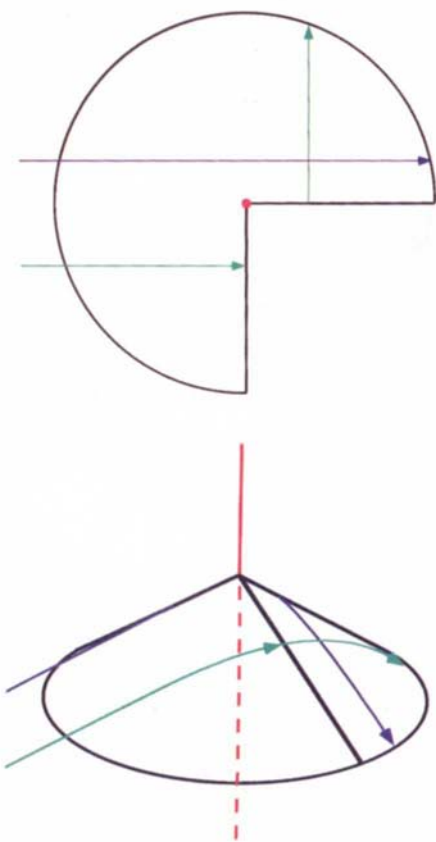


FIG. 1 The gravitational field of a cosmic string (red) gives space a conical geometry. At a fixed time, if the string runs vertically through the page, the cone may be visualized as a circular disk with a wedge removed (top). The sides of the wedge are then glued together (bottom). The green path is a continuous one that vaults the wedge. In the remaining diagrams, only the edges of the excised wedges are shown. Note that although the blue and green paths start parallel, they eventually converge and intersect.

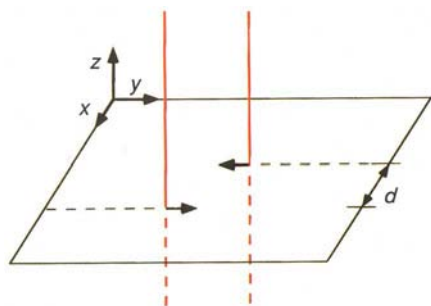


FIG. 2 A snapshot of Gott's two infinite strings (red) just before their closest approach (d , closest separation). In Fig. 3, the z direction is not shown.

a cosmic string is like a cone whose vertex lies on the string itself: a circle drawn around the cone has a circumference less than 2π times the distance to the vertex. Equivalently, one may imagine moving on a flat piece of paper with a wedge cut out, with the additional rule that any object travelling along the surface of the paper, when it hits the border of the wedge, instantaneously transports across the wedge to the other side, leaving at the same relative angle as it entered (Fig. 1).

This two-dimensional example is closely related to the four-dimensional cosmic-string spacetime. If one takes a cross-section of the spacetime through the string at a given instant of time, the cross-section of the string itself corresponds to the vertex of the cone (or of the missing wedge). An infinite stack of cones would correspond to an infinitely long cosmic string, where the vertices of the cones run along the string. The same would apply to an infinite stack of paper with a wedge removed from every sheet, and the vertices running along the string. Letting time evolve naturally brings back the fourth dimension of time.

The fundamental postulate of Einstein's special relativity is that the speed of light in vacuum is the same for all observers (unlike the speed of sound, which changes for an observer moving with respect to the background air). This leads to a loss of simultaneity: two events occurring at the same time as measured by one observer at rest occur at different times for a moving observer.

This has an important effect for moving cosmic strings. One can build a stationary string by stacking up sheets of paper with the wedges removed, plus the instantaneous-transport rule. But if the string is moving, the instantaneous-transport rule must change according to special relativity: points on opposite sides of the wedge on a single sheet are simultaneous only for an observer for whom the string is stationary. For an observer who sees the string speed by, the identified points on opposite sides of the wedge lie at different times. If the string is moving to the left, then trans-

porting across the wedge to the right results in a jump backwards in time (and transporting across the wedge to the left results in a jump forwards; see Fig. 3).

As mentioned before, Gott's time machine is based on two cosmic strings moving in opposite directions. If there were only one string, there would never be enough time to return to one's initial position and initial time (without going faster than the speed of light, which would violate the laws of special relativity). This can be seen most clearly from the point of view of an observer moving at the same velocity as the string, for whom the string is stationary and for whom there is no time jump at all. If there are no closed timelike curves for this observer, then there are no closed timelike curves for any observer.

With two cosmic strings moving in different directions the situation is altered, because no observer can see both strings at rest at once. A closed

timelike curve can easily be traversed by a time traveller who first moves around one string in the direction opposite to that string's direction of travel, then turns around and moves around the other string, opposite to its direction of travel, ending up at the starting point (the turnarounds appear in Fig. 3 as corners on the green curves; these accelerations are necessary because there are no closed timelike geodesics). If the strings are moving quickly enough, then because both time jumps are backwards, the observer can return to the same initial position and to the same initial time. The strings' speed necessary for this to work is related to the angle of the wedge, as the larger the wedge, the larger a time jump is accomplished. The actual relation between the two is that the speed v , as measured by an observer who sees the strings coming together with equal but opposite velocities, must be larger than $c \cos(\alpha/2)$ where c is the

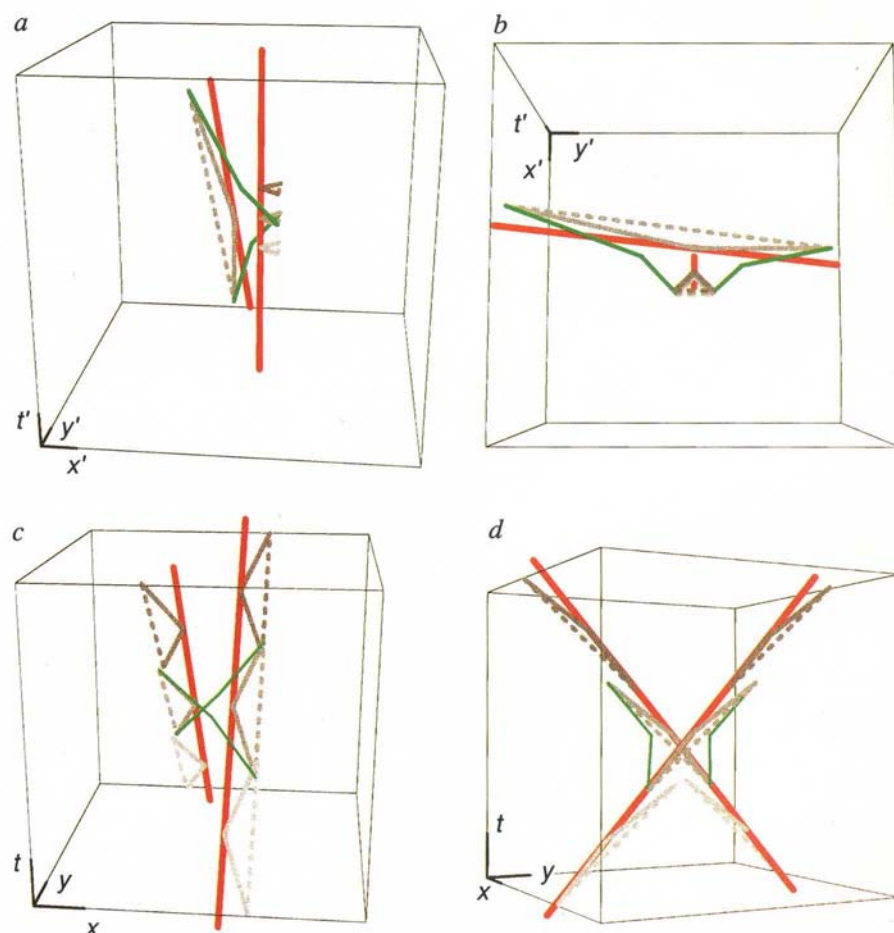


FIG. 3 Gott's time machine as seen by an observer at rest next to one of the strings (*a*, *b*) and by an observer who sees the two strings rushing together, with equal but opposite velocities, $0.89c$ (*c*, *d*). The time coordinate in each case runs directly upwards. The cosmic strings are red, the excised wedges are different shades of grey, and the closed timelike curve is green. Note that in *a* and *b*, the 90° excised wedges of the stationary string (right) are horizontal ($t' = \text{constant}$) surfaces. The wedges associated with the other string, which is moving, are stretched and its glued edges connect points at different times, because of the loss of simultaneity in special relativity. Owing to the effects of special relativity, the wedges in parts *c* and *d* are not surfaces of constant time (horizontal) for either string. Parts *b* and *d* are the same as parts *a* and *c*, respectively, but are seen from a different perspective.

speed of light. In our diagrams with $\alpha = 90^\circ$, v must be larger than $0.71c$ to make a time machine. In Fig. 3, the strings are moving with $v = 0.89c$.

The timing of a trajectory such as the one just described is critical: if the observer starts at the wrong place and time, time travel is impossible. Starting out near the point of closest approach of the strings, but either too early or too late, one cannot complete the trip at the initial time and place of departure without going faster than light. One may start far away from the point of closest approach, but then one must be going close to the speed of light to get there in time to circle both strings. Cutler⁴ has found explicitly which starting points allow travel along closed timelike curves and which do not. The regions where closed timelike curves exist form a collar around the location and time of closest approach (the central region of Fig. 3c, d). The boundary of the regions comes in and exits at the speed of light. In this sense, there are no closed timelike curves in the far past and far future of the spacetime. Furthermore, one could create closed timelike curves using strings which have travelled for only a limited period of time.

Headway

As cosmic strings are considered a realistic form of matter, and time travel would be quite an achievement, considerable effort has gone into looking for obstacles that might prevent one constructing or operating Gott's design. Headway has been made because a system of parallel strings moving in four spacetime dimensions is mathematically indistinguishable from a system of point masses interacting gravitationally in three spacetime dimensions. Exploiting this analogy (first pointed out by Gott¹²), Deser, Jackiw and 't Hooft show³ that, although the masses corresponding to each cosmic string move at less than the speed of light, what they call the total momentum (in three space-time dimensions) would correspond to a single string moving faster than light. Such systems are called tachyonic, from the Greek for fast.

It is not clear what the repercussions of this may be, but it does appear to be closely tied to the possibility of time travel. If the strings slow down enough to eliminate the closed timelike curves, the momentum of the system ceases to be tachyonic. One might think that it

would be enough to create a tachyonic subsystem in isolation, but this appears insufficient, because if any closed time-like curves exist, it may be shown that they are not confined to any finite region (in agreement with Cutler). Thus this work suggests that although the cosmic strings themselves are a reasonable form of matter, the combined system of two such infinite strings, moving at high relative velocity, might never be obtained in our Universe. These results are only suggestive, however, as the exact relationship between momentum in three spacetime dimensions and in four spacetime dimensions is still not clear. For example, in three spacetime dimensions the momentum of the system of strings is tachyonic, but the motion of the centre of mass of the system is non-tachyonic. In four spacetime dimensions they should be the same.

Using somewhat different methods, Carroll, Farhi and Guth² reach the same conclusion. They analyse a system containing several cosmic strings, again exploiting the parallel with a system of masses in three spacetime dimensions. The geometry of such a system is characterized by a universal quantity (known as the holonomy) which describes the way in which a vector changes direction after being carried around a closed curve. The change in direction is specified by a quantity related to the momentum of the combined system of strings, which Carroll, Farhi and Guth show corresponds to tachyonic matter (when closed timelike curves are present). They also examine several likely ways of creating systems with tachyonic momentum, but they cannot do so starting with non-tachyonic matter. Again, this suggests that Gott's time machine may be fundamentally out of reach.

There are even more obstacles. In constructing a mathematical description of physical systems, one generally makes idealizations of some kind. In a laboratory, cosmic strings of infinite length would not be available, but very long portions of strings might be used (or even two halves of a very long and almost-straight loop of cosmic string). Would Gott's time machine still work if the infinitely long cosmic strings were replaced with very long, but finite ones? Gott suggests that any closed timelike curves created with finite lengths of string would form a black hole and then fall into it before they could be exploited. The work by Carroll, Deser and their collaborators does not directly address this question, although they show that there is something suspect about the global geometry (related to the tachyonic nature of the system). Effectively, Gott's machine requires spacetime to have unusual large-scale geometry, and unfortunately for the

aspiring inventor, nothing one does in a laboratory can alter this large-scale geometry.

Conjecture

In a bold attempt to rule out time machines altogether, Hawking now puts forward what he calls "The Chronology Protection Conjecture"⁵. To support this grand conjecture, he has proved a theorem which shows that if one constructs a time machine out of very long but not infinite strings, then either one must surround the time machine with a shell of negative energy, or singularities of the same sort that one finds at the centre of black holes would form, shutting off access to the time machine (and destroying the laboratory in the process). This seems to offer a definitive answer to the question of whether one could build a time machine in a laboratory based on Gott's design, and whether it would work.

Unfortunately the answer is no (without resorting to the use of negative energy). Although Gott's design appears to be based on a realistic type of matter (cosmic strings), actually to build one that would not disappear into a black hole would apparently require large amounts of (unrealistic) matter with negative energy. Even so, if our Universe is infinitely large and infinite cosmic strings naturally formed after the Big Bang (as predicted by several cosmological models), then the possibility that the Universe itself created a time machine cannot be ruled out.

While there is still hope that one day a sufficiently clever design may make building a time machine possible, it is beginning to seem more and more improbable. Like the perpetual motion machines of the nineteenth century, the designs have an elegant simplicity (as well as enormous commercial potential), but it seems that Nature may abhor them just as much. □

Bruce Allen and Jonathan Simon are in the Department of Physics, University of Wisconsin, PO Box 413, Milwaukee, Wisconsin 53201, USA.

Correction

THE scanning tunnelling micrograph of fullerene molecules by Y. Zhang *et al.*, which appeared in News and Views recently (*Nature* **356**, 383) first appeared in the *Journal of Physical Chemistry* (**96**, 510–513; 1992), not the *Journal of the American Chemical Society*, as reported.

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Travelling by TRAM

Peter Walter

THE mechanism by which proteins move across the membrane of the endoplasmic reticulum (ER) is still obscure. Not only do soluble, hydrophilic proteins need to be moved completely across the lipid bilayer, but membrane proteins need to be integrated in their proper, asymmetric orientation. For proteins that have many membrane-spanning domains, the process requires the correct insertion of each transmembrane domain. These events are catalysed by ER membrane proteins which are thought to interact during their functional cycle and to constitute a multicomponent translocation apparatus, termed translocon. On page 47 of this issue¹, Rapoport and colleagues describe their identification of a hitherto unknown ER membrane protein and provide evidence that it is a component of the translocon.

The translocon mediates the binding of ribosomes engaged in synthesizing secretory or membrane proteins that have been targeted to the ER membrane by the signal recognition particle and its receptor. After targeting, translocation of the nascent proteins presumably proceeds through an aqueous pore² that may be shielded from the hydrophobic core of the membrane by the translocon components.

It is likely that the translocon is a multifunctional and perhaps dynamic assembly. Beyond a basic set of components required for translocation itself, different translocating proteins have different requirements, such as signal peptide cleavage or glycosylation, that may necessitate the incorporation of additional components into the translocon. Furthermore, elements in the translocating proteins, termed topogenic sequences, can regulate steps in translocation³. Stop transfer sequences, for example, will halt the translocation of nascent protein chains and thus lead to their integration into the membrane. These sequences appear to be recognized by translocon components and, interestingly, remain in contact with the translocon until protein synthesis is terminated⁴. Translocon components must also promote the integration of signal anchor sequences in the proper orientation which can give rise to either type I or type II membrane proteins.

To understand this functional complexity, much effort is currently being spent to identify the protein components of the translocon. One powerful approach has been to identify molecules that can be crosslinked to nascent chains during their transit across the ER membrane. It has been shown that the cross-

linking patterns are complex, indicating that the nascent chains lie close to a number of different glycosylated and nonglycosylated ER membrane proteins⁵⁻⁸.

In pioneering studies, Krieg *et al.*⁵ and Wiedmann *et al.*⁸ identified ER membrane proteins to which short nascent chains could be crosslinked. Prominent among these were membrane glycoproteins with a relative molecular mass of 35,000 to 39,000. Wiedmann *et al.* termed a major crosslinking target SSR α (SSR standing for signal sequence receptor), on the assumption that the crosslinks were specific to the signal sequences of translocating chains. They proceeded to purify a major ER membrane glycoprotein and, assuming that this protein was the only ER membrane glycoprotein of this size range, referred to it as SSR α . Both assumptions, however, quickly proved to be problematic. First, not only the signal sequence, but also distant parts of the translocating

nascent chains can be crosslinked to the membrane glycoprotein⁵. Thus, the crosslinked protein does not function as a 'signal sequence receptor'. Second, antibodies to SSR α precipitate only a very small proportion of the crosslinked products, suggesting that another glycoprotein, not SSR α , is crosslinked to the nascent chains⁵. Finally, in a carefully controlled study, Migliaccio *et al.*⁹ showed that reconstituted ER vesicles that have been immunodepleted of the SSR α and its associated subunits show no detectable defect in protein translocation of both secretory and membrane protein substrates.

Taken together, these findings make it unlikely that SSR α plays a vital role in ER protein translocation. As SSR α can be crosslinked to longer nascent chains protruding into the ER lumen^{1,10}, it remains possible, however, that SSR α is involved in aspects of the maturation of the nascent chain which are not monitored or which are not rate limiting in the *in vitro* translocation assay.

In a remarkably complete study, Görlich *et al.*¹ now provide evidence that a different ER membrane glycoprotein, TRAM, participates in protein transloca-

Herschel and hypo

LIKE the best of his era, John Herschel (1792–1871) was a polymath. The son of the astronomer William Herschel (discoverer of Uranus) he founded the observatory in Cape Colony, South Africa, to map the southern skies, and showed the eponymous Magellanic clouds to be collections of stars. This work and his many other endeavours — in mathematics, chemistry, poetry and, following in Newton's footsteps, as Master of the Mint — are the subject of a bicentennial celebration at the Royal Society next week (13 May). It was his experience in chemistry that allowed Herschel to make a decisive contribution to the development of photography — the fixing process. Fox Talbot had developed a 'photogenic drawing' method using paper treated with silver salts. The unexposed areas could be only partially protected by sodium chloride solution, which slowed down but did not halt the light-induced darkening of the silver salts. By showing that sodium thiosulphate ('hypo') dissolved away the unexposed areas, Herschel made permanent images possible. He also coined the terms positive and negative, invented the blueprint process (cyanotypes) and introduced photography to astronomy.

Contact negative of an engraving, fixed by hypo, made by Herschel on 5 August 1839 (the year Talbot and Daguerre each described their photographic methods).

IMAGE
UNAVAILABLE
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REASONS

R. P.

tion. TRAM was identified and purified as a crosslinking target of nascent protein chains that are translocating across the ER membrane (hence its name: translocating chain-associating membrane protein). It is likely that TRAM is identical to the main crosslinking target identified in the initial studies^{5,8}, but may have been confused with crosslinks to SSR α which is of similar size. Görlich *et al.* isolated and sequenced a complementary DNA clone and raised antibodies against a peptide predicted to comprise a portion of the cytoplasmic tail of TRAM. The authors then demonstrated that these antibodies efficiently precipitated the crosslinked product containing the nascent chain. This shows convincingly that the cDNA clone encodes the protein identified by the crosslinking approach.

To address the functional significance of TRAM during translocation, Görlich *et al.* depleted TRAM from ER vesicles. Study of protein translocation across the ER was revolutionized by Nicchitta and Blobel¹¹, who showed that reconstituted artificial lipid vesicles containing protein from a detergent extract of completely solubilized ER membranes are competent for translocation. Görlich *et al.* modified this procedure to improve the yield of sealed vesicles after detergent removal. Using this modified protocol, they removed TRAM by immunoabsorption from the detergent extract and then reconstituted TRAM-depleted vesicles. Translocation of two secretory proteins, β -lactamase and prepro- α -factor, was completely blocked in TRAM-depleted vesicles; surprisingly, however, translocation of a third substrate, preprolactin, was still observed, albeit at reduced efficiency. Readdition of purified TRAM to the immunodepleted extract restored the activity of the resulting membrane vesicles. So it seems that TRAM is required for ER translocation of at least a subset of proteins.

At present, TRAM is the most convincing candidate for a component of the mammalian ER translocon. Because the initial crosslinking studies showed that the environment in which a nascent

chain traverses the ER membrane is complex, it is likely that many more translocon components will be discovered. Genetic studies have led to the proposal that three interacting ER membrane proteins, Sec61p, Sec62p and Sec63p, are involved in protein translocation in yeast¹². One might predict that translocon components have been evolutionarily conserved, as is true for the signal recognition particle and its receptor¹³. Mammalian homologues of

Sec61p, Sec62p and Sec63p and a yeast homologue of TRAM may therefore be forthcoming. To date, different experimental approaches may only have revealed the tip of an iceberg in either system. □

Peter Walter is in the Department of Biochemistry and Biophysics, School of Medicine, University of California San Francisco, San Francisco, California 94143-0448, USA.

MICROBIAL GENETICS

Sexual identity and smut

Richard Egel

THE patterns of sexual reproduction in eukaryotic microorganisms are full of odd nooks and crannies. Yet, bit by bit, common schemes with lesser variations are emerging. Two remarkable papers in *Cell*^{1,2}, produced by Regine Kahmann's group in Berlin, take that process a stage further. They describe the molecular organization of mating-type genes in *Ustilago maydis*, the common corn smut, and show that one locus specifies complementary pairs of pheromones and receptors, whereas another locus with several alleles encodes two correlated subfunctions for each allele. These alleles are involved in self/nonself recognition.

Sexual differentiation need not be different from other kinds of differential gene expression, in which cytoplasmic states and environmental stimuli feed back on various controlling signals upstream of particular genes. Quite often, though, genomic differences mark the very top of regulatory cascades that govern sexual identity. This development can culminate in recognizable sex chromosomes, or heterosomes (as opposed to the remaining autosomes). But in primitive organisms more limited differences at the so-called mating-type loci are the norm.

In many fungi, such as *Saccharomyces*³, *Schizosaccharomyces*⁴ and *Neurospora*⁵, the mating-type regions encode regulatory proteins, which, on their own or in combination, control three sets of other genes; these genes are responsible more directly for the physiological activities during zygote formation or meiosis, or both. Two sets of such subordinate gene functions are limited to the haploid phase, one for either mating type, whereas the third set depends on heterozygosity and is therefore specific for the diploid stage.

In *U. maydis*, allelic differences are required at two loci (*a* and *b*) for the fungus to proceed through an entire life cycle. The mating-type locus in a strict

sense (*a*) has two alleles, but there is no recognizable DNA homology between the mating-type-specific segments of about five and eight kilobases, respectively⁶. Bölker *et al.*¹ now show that each one of these *a* alleles carries a gene for a mating pheromone and another gene for the receptor that is responsive to the opposite pheromone (see figure). (Such genes in yeast, for example, are common to both mating types, but their expression is separately controlled by the mating-type genes.)

Mutual stimulation by the pheromone response precedes fusion of two haploid, yeast-like cells of *Ustilago*. Thereafter, at the functionally diploid level, one might expect there to be no further need for the pheromones, but they nonetheless remain necessary for proper development. Cell fusion in most fungi, however, does not lead to a proper diploid stage; rather, the still-haploid nuclei exist in a common cytoplasm and establish a so-called dikaryon, their concerted mitotic divisions following a stereotyped and somewhat contorted pattern. Each cell division at a hyphal tip is accompanied by retrograde fusion of a tiny branch cell with the newly arisen penultimate cell, forming a so-called clamp connection and conveying one of the daughter nuclei backwards. In this way, both the growing tip and the new penultimate cell retain pairs of nuclei with different mating types, and an ordered pheromone response is probably required for the formation of each clamp connection.

The *b* locus, on the other hand, is important only after the initial fusion event, for maintenance of the dikaryotic state. This is also the infectious phase of smut as a plant pathogen. Some 30 different *b* alleles are known, and all heterozygous combinations of them are compatible whereas all homozygous combinations are not. It has long been a puzzle how molecular interactions at the protein level could account for such a

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pattern of combinatorial restraints. The partial cloning and sequencing of several different *b* alleles^{7,8} did not directly clarify that issue.

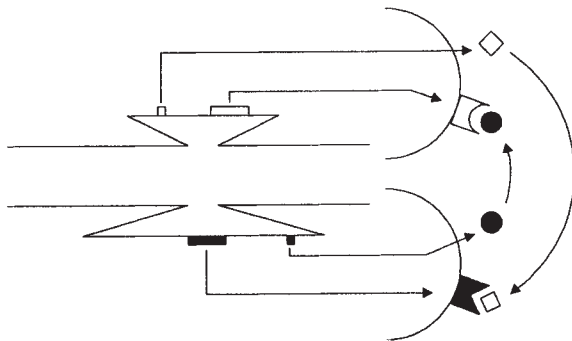
Four *b* alleles have now been sequenced more comprehensively by Gillissen *et al.*², and the results are revealing. It turns out that each so-called allele contains two functional genes with unrelated primary sequences but a similar tripartite organization: a constant part, a homeobox domain and a variable region. In memory of the once-divided city, one gene is called *B_n-E* and the other *B_n-W*. These findings support a new and convincing model for heterodimeric proteins (East and West united), in which all 'homoelellic' dimers have co-evolved for inactivity on account of a particularly fitting configuration, whereas this fit is broken by any formation of heteroallelic dimers. The presence of homeobox domains implies that these DNA-binding proteins have a regulatory function, although the potential target genes have not yet been characterized.

In most cases where mating pheromones and corresponding receptors have been identified, such components only occur in pairs per species. But there are notable exceptions. The ciliate protozoan *Euplotes raikovi*, for example⁹, has a series of *mat* alleles, each of which directly controls production of a specific pheromone, and a receptor too. These receptors, however, are not entirely specific for only one pheromone. Rather, they can bind to any pheromone, but they are specifically neutralized by the pheromone of their own *mat* allele and activated by any other kind. This, at least, is the most convincing explanation of the data, and it is formally analogous to the series of *b* interactions in *Ustilago*.

More and more examples of such structural differences at mating-type loci are being identified. Evidently, the ordinary meaning of 'alleles' is blurred when two (or more) functional genes show up at the same locus or map position, and 'idiomorphs' has been suggested as a substitute^{5,6} term. (Originally, the different alleles of ordinary genes were even termed 'allelomorphs', but that designation did not catch on.) On the other hand, alternative clusters of functionally related genes can act as units in their own right, and analogous configurations have occasionally been referred to as 'supergenes'. The driving force seems to be co-evolution of complementary subunits. Because free re-

combination would disturb this process, the clustering of those genes by rearrangement, together with elimination of corresponding DNA homology from the partner chromosome, has led to the highest degree of so-called linkage disequilibrium.

In the special cases of mosaic chromosomes involved in sex determination, it should be a natural extension of previous usage to talk of 'heterosomal' segments or regions, as opposed to the 'autosomal' regions that are common to both sexes



Structure and function of the *a* locus in *Ustilago maydis*. The two *a* alleles resemble insertions, with unrelated sequences of DNA, occupying allelic positions within otherwise homologous chromosomes. The mating-type-specific segments are here termed heterosomal regions. Either one of them carries a short gene for a secreted mating pheromone, as well as a longer gene encoding a membrane-bound receptor which is specific for the opposite pheromone.

(or all mating types) of a species. This may even apply to mammalian X and Y chromosomes. When their mosaic nature was discovered, it was felt so astounding that the common region close to one telomere was termed 'pseudo-autosomal'.

The neatly worked out *Ustilago* story has now been added to the growing number of instances of heterosomal alternatives, and it may well become a textbook example. The best documented cases of linkage disequilibrium are self and nonself recognition systems, and the situation at the *b* locus in *Ustilago* is particularly reminiscent of linkage disequilibrium at mammalian immunoresponse loci. □

Richard Egel is in the Institute of Genetics, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark.

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DAEDALUS

Thick skin

THE depleted ozone layer is an increasingly uncertain shield against solar ultraviolet light. Daedalus now has a second line of defence. He points out that some dyes, such as acriflavine and gentian violet, stain the skin permanently. They don't wash off; you have to wait until the skin, in its slow process of self-renewal from below, sheds the stained cells from its surface.

So Daedalus is seeking a permanent, colourless but ultraviolet-absorbing skin stain. Some sunscreen preparations use a gallic-acid ester derivative to absorb the ultraviolet. Daedalus recalls those widely distributed natural gallic esters, the vegetable tannins. They bind powerfully to skin proteins — hence the traditional tanning process, which converts perishable animal skin into tough, flexible leather. Accordingly, DREADCO chemists are devising a tannin derivative which binds firmly to human skin and absorbs ultraviolet light. Formulated into DREADCO's new 'Body Armour' bath salts, it will stain you with a lasting ultraviolet-absorbing layer. The absorption may 'tail' slightly into the visible, giving the pleasing impression of a slight natural tan.

Regular bathing with 'Body Armour' tannic bath salts will not merely protect you from the ravages of the ultraviolet. It will also tan you like leather, toughening the outer surface of your skin. You will notice nothing. Tough but not brittle, the thin case-hardened surface layer will still flex easily on the supple tissue beneath. But you will now be armoured against cuts, abrasions, and insects. Mosquitoes, hornets and wasps will bend their stings vainly against your resistant hide, while biting insects such as fleas and lice will recoil in bafflement and distaste. For vegetable tannins are one of the plant world's main defences against insect attack. By binding to proteins they sabotage an insect's chemistry; they denature its masticatory and digestive enzymes and ruin its appetite.

Body Armour may even protect you from allergies. Many allergies are blamed on the house dust mite. This tiny insect-like creature shares our houses and feeds on our shed skin. Its gut enzyme, spread about in its faecal dust, can cause many distressing allergic reactions, including dermatitis. (Daedalus reckons this is a cunning evolutionary adaptation to increase the mites' supply of shed skin). The allergen can be inactivated by spraying a solution of tannin around the house. But the toughened, tannic skin shed by a Body Armoured household will solve the whole problem. It will denature the enzyme inside the mites, giving them terminal indigestion. David Jones

Sea-level rise or fall?

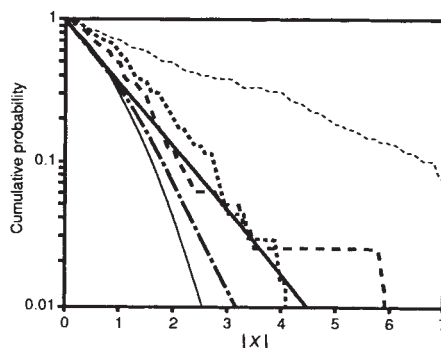
SIR — Schneider¹ provides just one example of how a change in the accepted values of parameters in climate-change models can completely alter the conclusions, turning a predicted sea-level rise into a predicted fall. If a model predicts a sea-level rise of 33 ± 32 cm in the year 2050 (ref. 2), what is the probability of an extreme rise of 150 cm? Or, for example, how should one interpret the 'confidence interval' defined by low- and high-growth schemes for future changes in population and energy production? Such questions can be addressed by comparing the data on the history of previous measurements in other fields and corresponding uncertainty estimates with the 'true' or 'best guess' values obtained later.

A convenient measure of the deviation of 'new' values from the 'old' values is $x = (a - A)/\Delta$, where a is the exact value, A the previously measured value, and Δ the old standard deviation. We analysed 79 elementary particle properties (mainly meson masses and lifetimes; thick dashed line in the figure), 69 forecasts of the primary energy demand for the United States projected for the year 2000 (thick dotted line) and the predictions of population for 133 countries for the year 1985 made in 1973 (thin dotted line, assuming that high and low estimates encompass 50% confidence interval)³. The cumulative probability distributions are shown in the figure together with the gaussian curve (thin solid line), which obviously grossly underestimate probability of large deviations. Also plotted is the Student's distribution for 10 degrees of freedom (heavy broken dashed line), illustrating the maximum effect of the finite sample size for energy forecasts (11 points per bin, or 10 degrees of freedom). A better fit to the data is obtained with a simple exponential distribution, $e^{-|x|}$ (heavy solid line).

This asymptotic behaviour appears naturally in a compound distribution where both the mean and standard deviation are independent, normally distributed, random variables. Following ref. 4, we assume that the calculated standard deviation Δ' is distributed around its true value Δ ; we denote this distribution by $f(t)$, where $t = \Delta'/\Delta$. If for simplicity, we assume $f(t)$ to be asymptotically gaussian, that is, $f(t) \sim \exp(-t^2/2\delta^2)$ as $t \rightarrow \infty$, and consider only the asymptotic behaviour of the probability distribution $P(x)$ when $|x| \gg 1$, it is straightforward to show that for $|x| \gg 1$, the probability distribution is not gaussian but exponential: $P(x) \sim \exp(-|x|/u)$. Here the new parameter u , $u = \delta/\Delta$, measures the unknown systematic component of the total

error and quantifies the uncertainty, δ , in the estimation of Δ . Gaussian and exponential distributions can be related by a single-parameter family of curves, so that the parametric uncertainty of current models can be quantified by analysing the record of earlier projections and estimating the value of u (ref. 3). From the figure, it emerges that $u \sim 1$ for physical constants and projections of energy demand, and $u \sim 3$ for models of population growth.

Fundamental physical constants are generally considered to be the most reliably known parameters, yet analysis of the trends in their measured values indicates widespread overconfidence in the completeness of our knowledge⁵.



Cumulative probability of finding a deviation greater than $|x|$. The data are drawn from various sources: projections of US energy demand in 2000 AD (heavy dotted line); measured parameters in elementary particle physics (heavy dashed line); projections for population in 2000 AD for 133 countries (thin dotted line). Theoretical curves for a gaussian distribution (thin solid line), Student distribution (heavy broken dashed line) corresponding to the sample size per bin for the energy data, and an exponential distribution (heavy solid line) with $u = 1$.

There is every reason to expect similar or greater effects in models of global environmental change. For sea-level rise, there is no long history of projections that we can use to estimate the value of the parameter u . Error estimates in this case are little more than educated guesses; if we conservatively assume $u=1$, in the 'business-as-usual' scheme the normal distribution places the probability in 2050 AD of extreme sea-level rise greater than 1 m at 0.5% (ref. 2) in contrast to the 5% probability based on an exponential distribution³, a difference of an order of magnitude. We

wish to encourage others to quantify the predictive capabilities of their models by using the historical trends in parameter values from previous studies.

Alexander I. Shlyakhter

Daniel M. Kammen

*Department of Physics and
Northeast Regional Center for
Global Environmental Change,
Harvard University, Cambridge,
Massachusetts 02138, USA*

Mirror script

SIR — Smetacek¹ explains the leftward ancient mirror writing of the Semitics and the archaic Romans and Greeks by an ancient use of the left hand. There is another explanation involving leftward eye movements, the left visual field and the right hemisphere.

Eye movements, the covert scanning of letters and mirror-image perception of words, are linked to the two visual fields. Leftward scripts (scanned with leftward eye movements) are read through a visual window extending into the left visual field (rightward ones are read through one extending into the right visual field)². Mirror-reversed words³ are better identified than their unreversed images in the left visual field, with the opposite being the case in the right field. Visual fields and eye movements are also connected to the two cerebral hemispheres. The left visual field is connected to the right hemisphere and the right with the left hemisphere — in part this explains the connection between mirror perception and visual fields: the two hemispheres in some circumstances represent visual⁴ engrams learnt in the other hemisphere in their mirror form. Also, each hemisphere controls eye movements directed in the opposite direction (the right hemisphere controls leftward eye movements and the left hemisphere rightward ones), so images scanned through the left visual field into the right hemisphere are also reciprocally controlled by this hemisphere.

Reading is a demanding ocular-motor activity, particularly for inexperienced (or archaic) readers. Ocular-motor control, at least for tasks that have not been over-learned, is a right-hemisphere specialization⁵. Thus in the early stages of reading development the ocular-motor requirement to scan writing would have produced a right-hemisphere bias to control reading eye movements with this hemisphere and so favour the rise of the leftward reading (and writing) over the opposite, rightward one. So I suggest that leftward writing originates not in the ancient use of the left hand but in the use of the right hemisphere to control the eye movements of early readers.

Why did writing direction change?

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The Greek alphabet represents vowels fully and consistently (phonological transparency), whereas the Semitic alphabet only partially represents them (phonological opaqueness). Phonological identification seems to be restricted to phonologically transparent writing (although it is not an obligatory process), whereas context-dependent processes have to be used to identify phonologically opaque words⁶. But phonological processing skills (like those of speech) are left-hemisphere lateralized. Thus the phonological reading of the Greeks probably induced a strong left-hemisphere involvement in reading and thus favoured the use of rightward eye scans and rightward writing. The evidence of ancient bidirectional writing suggests that this might have occurred gradually, with some shared hemisphere involvement in the archaic period, producing the coexistence of both writing directions together with unreversed mirror and reversed engrams.

This conjecture concerns only early leftward readers; therefore the existence of left-hemisphere literacy in modern leftward scripts such as Hebrew and Arabic does not necessarily conflict with it. Religions can fix writing because they forbid any form of alteration of sacred works (both the Torah and the Koran contain injunctions against changing them).

John R. Skoyles

Department of Psychology,
University College London,
Gower Street,
London WC1E 6BT, UK

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A disappearing speciation event?

SIR — Coyne points out¹ that there is still controversy as to whether two genetically distinct, geographical forms of a species (referred to here as races) are more likely to speciate when they are in contact or when they are in isolation. Races in contact may be considered less likely to speciate because the continuing flow of genes between them inevitably lessens the possibility of genetic divergence. But if the races making contact produce unfit hybrids then selection may be expected to favour alleles that reduce interracial matings and hence hasten speciation, according to the much-discussed 'reinforcement' model of Dobzhansky. Although there are theoretical grounds for believing that reinforcement is not a widespread process^{1,2}, the circumstances at a well-known contact between two

chromosomal races of house mice (*Mus musculus domesticus*) in northern Italy³ may be particularly favourable for this mode of speciation (see ref. 2). In this case, the races making contact have small populations and produce a single type of unfit hybrid.

The 'tobacco mouse' or Poschiavo race ($2n = 26$) geographically overlaps the Upper Valtellina race ($2n = 24$) along the valley of the river Adda near Tirano^{3,4}. The interracial hybrids have a complex heterozygous karyotype characterized by formation of a chain-of-five configuration at prophase I of meiosis, and hence would be expected to be predisposed to meiotic abnormalities and infertility⁵.

Until recently, only one site was known to contain both races. From 1978 to 1983, Capanna and Corti^{3,4} found 90 specimens of the Poschiavo race and 60 of the Upper Valtellina race, but no hybrids, in the small village of Migiondo. The two races were found in large numbers in the same building³ and laboratory studies of mice from this site indicated genetic and behavioural differences between the races and an inability to interbreed in captivity¹. We believe that the Upper Valtellina and Poschiavo races may have speciated very recently in Migiondo by the reinforcement process.

Over the past three years we have sampled the whole area of contact between the Upper Valtellina and Poschiavo races in great detail (214 animals, 39 sites; manuscript in preparation). In small samples from two villages, Sondalo and Sommacologna, we find both Upper Valtellina and Poschiavo race individuals and hybrids. We have also been able to cross mice from the two races in captivity and demonstrated that the hybrids do indeed suffer meiotic abnormalities, although they are usually not sterile (manuscript in preparation). However, among 37 mice from Migiondo, all were of the Poschiavo race.

There are numerous examples where different chromosomal races of house mice intermate successfully in the wild and in captivity⁵; the failure of the races in Migiondo to do so is, to our knowledge, unique. Therefore, we believe that the successful interbreeding of the Poschiavo and Upper Valtellina races demonstrated in our field and laboratory studies is the normal situation when these races come into contact in the wild. Presumably, therefore, when these races came together in the relatively isolated village of Migiondo, probably within the past few hundred years^{3,4}, hybrids were initially produced. If so, the lack of hybridization noted by Capanna and Corti³ would, of the alternative explanations available⁴, be

most reasonably ascribed to the reinforcement process. It is of further interest that one of the new species apparently became extinct thereafter, perhaps the most common fate for new species arising in small populations.

Heldi C. Haufler

Jeremy B. Searle

Department of Zoology,
University of Oxford,
South Parks Road,
Oxford OX1 3PS, UK

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New dimension for Mendeleev

SIR — The article "New dimension for Mendeleev" by John Maddox (*Nature* **356**, 13; 1992) about a suggestion of a third dimension for the periodic table by Leland C. Allen contains the statement that "the extra dimension may be half a century too late". In fact, essentially the same suggestion was made by me in 1939 in the first edition of my book *The Nature of the Chemical Bond* (Cornell University Press). A conventional two-dimensional periodic table is shown on page 54 (of the 3rd edition, 1960) and the third dimension is shown on page 94 by a figure showing the periods of the table with the elements indicated on the horizontal axis at their electronegativity values. The conventional table is useful in indicating the values of the elements and providing a basis for correlating other properties and the second table has additional usefulness, as was discussed in 1939 and later in my book.

Instead of the electronegativity, Allen uses a closely related quantity, the configuration energy, which is the first ionization energy of the neutral atom to the mean energy of the spectroscopic states based on the excited configuration. I had stated that the electronegativity is the energy of attraction of the atom in a stable compound for an outer electron; and then in 1934 Robert S. Mulliken expressed it as the mean of the first ionization energy and the electron affinity of the neutral atom. Allen's configuration energy ignores the electron affinity and introduces a special way of evaluating the first ionization energy. All these scales have essentially the same rational basis.

Maddox says that Allen is said to be

most pleased that his configuration rationalizes the metalloid band, from boron to antimony, which separates the metals from the nonmetals. This result is not new: more than 50 years ago I did this job, pointing out that the metalloids have electronegativity close to 2 (2 for B, 1.9 for Sb), metals have smaller values, and nonmetals have larger values. Many other properties can be related to the electronegativity.

Linus Pauling

Linus Pauling Institute of Science
and Medicine,
440 Page Mill Road,
Palo Alto, California 94306, USA

Earthquake scaling

SIR — Pacheco *et al.*¹ argue that the frequency–magnitude relation for large earthquake catalogues contains a break in slope at magnitudes corresponding to the finite depth of the seismogenic crust. Thus ‘small’ earthquakes scale differently from ‘large’ ones.

Perhaps the strongest evidence they found for this is the association of variations in this break of slope with tectonic province, with subduction zones implying a deeper schizosphere than transform margins, exactly as expected from the effect of temperature on constitutive laws based on friction or fracture. The question then remains, as Kisslinger² points out, how exactly are we to compensate for this in seismic hazard estimation? Moreover, can we do this in a way that brings in deterministic geological or tectonic constraints, on time periods much longer than the 100 years or so of instrumental recording of earthquakes?

At least one answer to this question already exists. Figure 1a shows a tectonically constrained fit to a form of the frequency–magnitude distribution suggested by myself and Burton^{3,4} applied to the Southern California Earthquake Catalogue for the time period 1932–72. The discrete frequency distribution has the form $\log(F) = a - b m - \lambda 10^{(c m + d)}$, where b is the seismic ‘ b -value’ and λ is a parameter that depends on the seismic moment release rate dM_0/dt . The seismic moment magnitude m used in the formulation is given by $\log M_0 = c m + d$.

The resulting cumulative frequency distribution $N(M \geq m)$ is calculated from this definition, and is shown in Fig. 1b. Although the latter figure is often cited in the literature as evidence for the scale-invariance of earthquakes throughout the magnitude range⁵, the original paper⁴ shows that strict scale-invariance may in fact break down at magnitude 6.5 or so, for reasons exactly similar to those proposed by Pacheco *et al.*

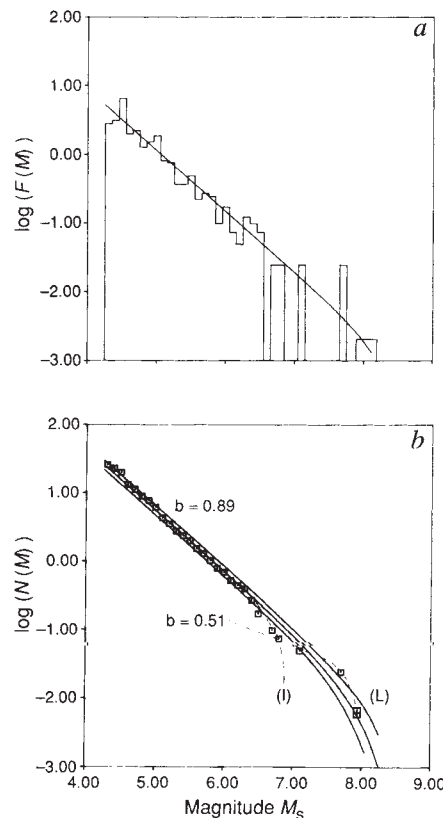


FIG 1 a, Discrete frequency–magnitude plot for the Southern California Earthquake Catalogue, from ref. 3. The curve is a fit to the discrete frequency distribution in the text, constrained by the average magnitude $\langle m \rangle$ and the seismic moment release rate dM_0/dt . In this case λ is small but positive. b, Cumulative frequency–magnitude plot. Both the best-fit curve and the (log normally distributed) errors are shown, together with the observed recurrence rates inferred from palaeoseismic trenching ($M_s = 8$). Intermediate (I) and large (L) earthquakes appear to follow separate distributions associated with different b -values, but despite this the moment-rate-constrained curve predicts the observed occurrence of the large events within its error bounds.

In essence, the technique uses a maximum entropy approach to derive the discrete frequency distribution, based on Shannon's information theory, to combine magnitude information from the earthquake catalogue with tectonic information from the slip rate, and the length and width of the seismogenic zone of interest. Thus the solid curves in Fig. 1 at high magnitudes are not just line fits to the magnitude data, but are also constrained at higher magnitudes to have a total seismic moment release rate equal to that observed over geological time in the area. The technique allows an equal, greater or lesser occurrence than predicted by extrapolation from the occurrence of smaller magnitudes (I in Fig. 1). This is important because in single tectonic provinces the extrapolation of the straight line portion tends to underestimate the occurrence of large

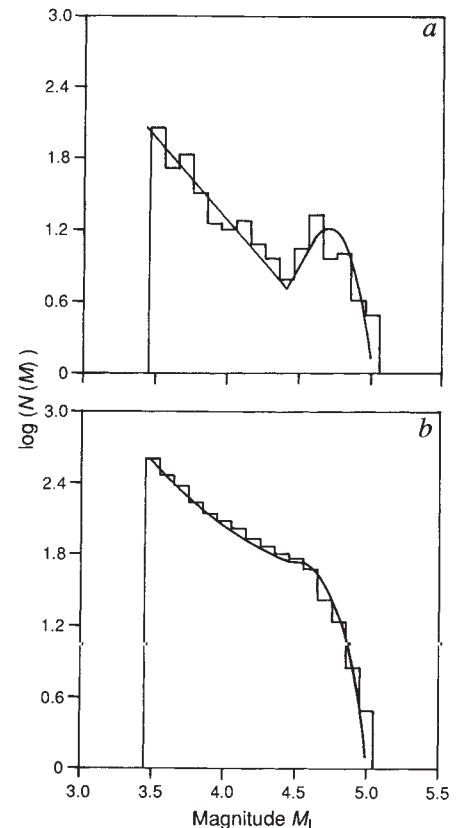


FIG 2 a, Discrete frequency–magnitude plot for the seismicity preceding the 18 May 1980 eruptions of Mount St Helens, Washington State. The curve fit to the data has a log-linear portion for small earthquakes, consistent with a power-law frequency–length distribution, and a peak at larger earthquakes, fitted in this case by a Gaussian frequency–length distribution. b, Cumulative frequency–magnitude plot of the same data set, fitted with the same curve.

earthquakes⁷, whereas an overestimate results in a larger, combined data set¹. Errors in the estimated slip rate, maximum magnitude and the moment–magnitude relation are explicitly included, as well as the statistical error in the curve fit at lower magnitudes. Covariance terms are included in the error matrix. As a result, the magnitude occurrence rate predicted using this technique by the slip rate on the San Andreas fault is in excellent agreement within these error bounds with the rate observed by palaeoseismic trenching⁶. In areas where the coupling between plates is less efficient, the technique places upper bounds on the hazard.

A corollary of the change in scaling hypothesis is that seismicity in volcanic provinces might be expected to have a break in slope at even smaller magnitudes, due to the raising of isotherms by magma injection. Figure 2 shows an example for the sequence of earthquakes preceding the eruptions of Mount St Helens⁸ in 1980. The cumulative frequency curve shows a concave upward slope at lower magnitudes, followed by

an inflexion at magnitude 4.5, and a concave downward slope thereafter. One might even be tempted to fit two straight lines to the data above and below M_L 4.5, if it were not for the fact that the discrete frequency-magnitude plot shows a gaussian peak (in the inferred length distribution) rather than a simple break in slope above this magnitude. The peak magnitude corresponds to a seismic source roughly equal to the 2 km depth of the seismogenic zone in the volume above the magma chamber. This figure also highlights the importance of plotting both discrete and cumulative frequency data before the type of curve fit is chosen in the first place.

Ian Main

*Department of Geology and Geophysics,
University of Edinburgh,
Grant Institute, West Mains Road,
Edinburgh EH9 3JW, UK*

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Electrical bursting in islet β cells

SIR — Ämmälä *et al.*¹ presented a novel theory to account for the bursting pattern of glucose-induced membrane electrical activity in pancreatic β cells. They show that exposure of clusters of cultured mouse β cells to carbamylcholine (CCh) or to dibutyl cyclic AMP (db-cAMP) triggers repetitive membrane hyperpolarizations. These waves of hyperpolarization are attributed to activation of low conductance calcium-activated K channels by the transient release of calcium from endoplasmic reticulum stores controlled by inositol 1,4,5-trisphosphate. The authors conclude that the burst pattern of β -cell electrical activity depends on this periodic release of intracellular calcium and on the presence of intra-islet hormones (such as glucagon) and neurotransmitters (such as acetylcholine). Although this theory may explain hyperpolarizations in their cultured β cells, the relevance of these observations to the electrophysiology of β cells in intact islets of Langerhans is not apparent.

First, rather than potentiating β -cell responses to glucose, Ämmälä *et al.* find that both db-cAMP and CCh hyperpolarize the membrane and inhibit membrane electrical activity. The depo-

larizing and potentiating effect of these, or pharmacologically comparable, agents on β -cell electrical activity has been well established by several groups^{2–7}. A well-documented alternative ionic mechanism for muscarinic effects⁸ is not discussed by Ämmälä *et al.*

Second, Ämmälä *et al.* point out that the intracellular calcium transients and K-channel activation are independent of membrane potential and calcium influx, yet they propose to explain a bursting mechanism which is clearly voltage-dependent in intact mouse islets. As recently reviewed⁹, electrical stimuli as brief as 50 ms during a burst of spikes interrupts the burst and triggers an all-or-none silent phase indistinguishable in voltage trajectory and duration from endogenous silent phases. Similarly, stimuli as brief as 1 s during silent phases triggers all-or-none bursts of spikes. In both cases, the subsequent bursting rhythm is immediately and completely reset by these brief electrical stimuli. It is not evident how voltage-independent calcium release from intracellular stores could possibly explain this clear voltage-dependence of bursting.

It is difficult to reconcile the mechanism proposed by Ämmälä *et al.* with the published observations and, hence, the importance of the proposed mechanisms for pancreatic β -cell electrical activity and insulin secretion is questionable. It is disappointing that the cultured mouse β -cell preparation, the only culture system capable of intermittent electrical activity, appears to achieve bursting by a mechanism unrelated to that used by β cells in intact islets.

Daniel L. Cook

*Division of Metabolism (151),
Seattle VA Medical Center,
1660 S Columbian Way,
Seattle, Washington 98108*

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RORSMAN AND BERGGREN REPLY — Although CCh and cAMP analogues are normally depolarizing in intact pancreatic islets, repolarizing effects — particularly at high concentrations — have been reported^{1,2}. It is possible that the failure of CCh, for example, to exert a stimulatory action in the cultured β cells results from the loss of the ion channels (probably Na^+

conducting³) mediating this response and the inhibitory action prevails.

As to Cook's second point, phospholipase C, the enzyme catalysing the production of InsP_3 , is Ca^{2+} -dependent in the β cell⁴. The Ca^{2+} influx occurring during electrical activity can consequently be envisaged to result in stimulation of InsP_3 formation, mobilization of intracellular Ca^{2+} and activation of K^+ channels, thus providing a direct connection between regulation of intracellular Ca^{2+} stores and electrical activity. Another mechanism that may be significant in this context is Ca^{2+} -induced Ca^{2+} release⁵. The observation that the oscillatory rhythm in intact pancreatic islets can be reset by current injections⁶ does therefore not exclude the involvement of intracellular Ca^{2+} pools in regulation of membrane excitability in the β cell.

We disagree with Cook's statement that the proposed mechanism is "unrelated to that used by β cells in intact islets" because the mechanism evoking bursting in intact islets is not known. Many processes are thought to be involved, for example inactivation of the Ca^{2+} current^{3,6} and variations of the activity of ATP-regulated K^+ channels⁷. Although such processes may well be important, direct experimental evidence for their involvement has not yet been provided. On the other hand, activation of the current we described in our paper⁸ clearly represents one mechanism capable of producing membrane potential oscillations in cultured β cells. Considering that the hormones and neurotransmitters required for its activation are present and released within the pancreatic islet, it seems reasonable to assume that this mechanism is operating in the intact tissue and thus contributes to the bursting pattern. Mechanisms similar to those we reported for the β cell have now been demonstrated to underlie rhythmic hyperpolarizations also in pituitary cells⁹.

Patrik Rorsman

*Department of Medical Physics,
Gothenburg University,
S-41390 Gothenburg, Sweden*

Per-Olof Berggren

*Department of Endocrinology,
Karolinska Institute,
S-10401 Stockholm, Sweden*

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Counsel of despair

Brian Pippard

Understanding the Present: Science and the Soul of Modern Man. By Bryan Appleyard. Picador: 1992. Pp. 283. £14.99. To be published in the United States by Doubleday early next year.

BRYAN Appleyard is unhappy. Living, as I suppose he does, in reasonable comfort and having not just the opportunity but the professional duty as a journalist in the world of ideas to think about higher things, he recognizes both the material well-being of Western society and its lack of moral conviction. Because both, in his view, are engendered by science, he wishes there were a way of remedying the one without impairing the other.

In this well-written book, published tomorrow, he wastes little effort on the positive achievements of technology, but settles down with a will to his indictment of pure science. It is hard science that takes the knocks, especially physics as typified by the public figures who recount with breathtaking assurance the whole history of the cosmos from birth to death, and promise the end of Real Science when a Theory of Everything is brought forth.

If everything meant only the material world it would not be so bad, but it also includes the human mind, and sometimes even poor old God who appears to have had little choice in the sort of universe He would create. I wish one could say that Appleyard has simply been listening to the wrong pundits, yet this sort of triumphalism is unfortunately widespread. All too rarely do I find colleagues who will assent to the proposition (which I find irresistible) that the very ground-rules of science, its concern only for public knowledge, preclude its finding an explanation for my consciousness, the one phenomenon of which I am absolutely certain. Mostly they admit indeed that it will be a tough job, but like to believe that in due course the relationship of consciousness to brain activity will be made clear, and the ghost in the machine exorcised.

Such excess of confidence is a fairly recent development, although the exaltation of science as the key to heaven on Earth is an old story and has been attacked on many occasions. C. S. Lewis's *Silent Planet* trilogy offers the nightmare of a virtually irresistible science-based conspiracy, and the honest scientist's vulnerability to evil temptation, if presented as an intellectual challenge; but few readers who sympathize with the message will be consoled to find the happy ending (for all but the wicked) engineered by supernatural powers.

Aldous Huxley's *Brave New World* in its bleak pessimism is a better blueprint for Appleyard's fears, the surrender of the soul in the midst of plenty. In Appleyard's own words, "Science made us, science broke us" — it has taken away our ancient faith and given nothing in return. He tries hard to find merit in

... it is not true that science has cut away the roots of revealed religion. That is beyond its power ...

alternative scientific creeds but cannot in the end accept them. Environmentalism, anthropic principles, the hybridization of Eastern mysticism with physics, morphogenetic fields and 'implicate wholeness' are all found wanting as means of spiritual rebirth, because one cannot arbitrarily choose to build a religion out of an idea; it must spring from a deeper source — the thirst for understanding not just how, but why. If one has been persuaded that science, the only purveyor of truth, can at best tell one how, and with the aid of philosophy has denied the validity of 'why' questions, one will find no satisfaction in a contrived surrogate.

But, whatever individual scientists may say, it is not true that science has cut away the roots of revealed religion. That is beyond its power, though its legitimate critiques have starved the roots of material nourishment and thus weakened or destroyed the faith of the merely credulous. I cannot accept that it is a fault in scientists that they have worked hard to eliminate demonstrable errors, although it is perhaps a fair charge that they have frequently tackled the job with uncharitable gusto. There are those, however, who have gone further and assumed authority, in the name of science, to brand all belief as superstition. They have abandoned healthy scepticism in favour of bigotry, and (if Appleyard's reading is accepted) the whole of science is reaping the reward. The cynical materialism they helped to foster begins to take for granted that science shares its own venality; and the rare but sensationally publicized cases of scientific fraud are taken as evidence of

endemic corruption. But this can give no satisfaction to the enemies of science, if the role of spiritual guide is to be taken over by a proliferation of mindless pseudoreligions.

It would be wrong, however, to leave the impression that Appleyard sees the source of the trouble to lie solely in the antireligious excesses of a few enthusiasts. It is rather the whole scientific enterprise, from Descartes and Galileo on, that has done the damage, with philosophical demagogues such as Bertrand Russell, the archenemy of metaphysics, leading the demolition gang.

Appleyard seems to me to have allowed himself to be argued into the sort of despair that can thrive only in a leisure society, and which is entirely irrelevant to the generality of decent people who love their kind, and work for them, and only rarely wonder if life is worth living. The scientists among them, and there are plenty, need feel no shame at being part of a historical development that cannot be undone. They have a perfect right to be proud of the process and to enjoy it for its intellectual beauty.

This beauty is not the same as the awe that popularizers try to inspire with their grandiose stories of the cosmos, but is to be found all around us in simple things whose appreciation demands little in the way of advanced theory. Such understanding is no enemy of faith, and we should surely be trying to help a new generation of children grow up sharing our pleasures, or at least sympathizing with them. Exploratories are an excellent start, although the solemn, not to say pedantic, emphasis on introducing formal structures into the curricula at too early an age can easily undo the good work. Producing only experts is not a sound primary aim — we need a scientifically literate (or semiliterate) population from which the experts select themselves without being regarded as priests or pariahs. Ideally, everyone should be encouraged to feel involved, even if marginally, in a venture that combines enjoyment with mental discipline and a rigorous ethic. These features may not add up to a religious sense of purpose, but they can serve as a pretty good substitute for those who have been denied the gift of faith.

"I have tried too in my time to be a philosopher", said Oliver Edwards to Dr Johnson, "but cheerfulness was always breaking in." We might do worse than take Mr Edwards as the patron saint of natural philosophy. His is a better gospel than despair; and what fun we should have spreading it. □

Sir Brian Pippard, emeritus professor of physics in the University of Cambridge, is at the Cavendish Laboratory, Madingley Road, Cambridge CB3 0HE, UK.

Avian affairs

Mark Kirkpatrick

Sperm Competition in Birds: Evolutionary Causes and Consequences. By Tim R. Birkhead and Anders P. Møller. Academic: 1992. £39, \$75 (hbk); £19.50, \$39.95 (pbk).

THE so-called 'monogamous' birds are not the paragons of Victorian sexual sensibility for which they were once taken. This realization, growing over the past decade, has been confirmed by recent molecular studies showing that a substantial fraction (sometimes an outright majority) of chicks in many species are the offspring of males other than those tending their nests. With these and other seedy aspects of avian family life now exposed, there is a lot of interest in understanding their evolutionary implications.

In *Sperm Competition in Birds* Tim Birkhead and Anders Møller review the data and hypotheses on male-male reproductive competition in birds. The title might suggest something narrower, and in fact there is little evidence that the sperm themselves have elaborate adaptations for competitive chicanery. But competition for fertilizations has far-reaching effects, and the authors argue that it has shaped many aspects of behaviour and morphology. One dramatic example is the suggestion that male-male reproductive competition is the driving force behind the evolution of territoriality in birds. The book should be read by all who are interested



Greylags before the storm. This painting is taken from *The Art of Peter Scott: Images from a Lifetime*, which contains more than 150 superb colour reproductions of the work of this leading wildlife painter and conservationist, who died in 1989. Published by Sinclair-Stevenson, price £25.

in bird behavioural ecology.

Birkhead and Møller have produced a field guide to the hypotheses and gold mine of data. As yet, there are few convincing resolutions to the harder questions, but the subject is still young. At present, it is possible to test many of the arguments only by seeing if they are consistent with the data, rather than by showing that all reasonable alternatives can be ruled out, and the available comparative analyses do not always include rigorous controls for phylogenetic effects. Interesting patterns do emerge where it has been possible for the authors to overcome these limitations. Take, for example, two of the ways in which males decrease the odds that their mate will be fertilized by another male. One is mate guarding, in which males follow their mates closely during the fertile period, and another is frequent copulation, in which males displace the sperm of interlopers with their own sperm. The authors show convincingly that an evolutionary increase in either of these behaviours is correlated with a decrease in the other, indicating that the behaviours represent evolutionary alternatives.

A widely discussed question that is addressed by Birkhead and Møller is why some females solicit matings with many different males. (The corresponding question for males is not as controversial: more fertilizations presumably translates to greater fitness.) The authors consider in detail several possible costs and benefits of this behaviour. A clear difficulty is that none of the explanations seem to account well for the variation within species. My favourite explanation, for example, had

been that females benefit by insuring against an infertile mate. The authors point out, however, that not all females mate with more than one male, even in polygynous lekking species, where females have more social freedom than they do in many 'monogamous' species. If insurance against male infertility was an important consideration, one would expect almost all females to mate with several males, but only a minority seem to. Various costs to the behaviour (for example, the female's mate may withhold parental care from the offspring if he finds out that she has mated with others) likewise fail to explain the variation between females.

This situation is common in behavioural ecology, where many issues involve understanding the causes of individual variation. There are two genera of obvious explanations. The first is that each female is acting optimally, and that different circumstances make different behaviours ideal for different individuals. Probably most behavioural ecologists begin work with this view. A second possibility is that the variation is noise, maybe a side effect of hormone levels that reflect background environmental and genetic variation. This would be the favoured sort of explanation if one were discussing, say, a morphological trait such as tarsus length rather than a behaviour. Sorting out the relative merits of these two kinds of explanation will be a large part of the agenda for the behavioural ecologists who take on the many questions raised in the book. □

Mark Kirkpatrick is in the Department of Zoology, University of Texas, Austin, Texas 78712, USA.

New Journals issue

This year, *Nature's* annual New Journals review supplement will appear in the issue of 1 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

■ Journals that first appeared during or after June 1990 and issued at least four separate numbers by the end of April 1992 will be considered.

■ Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.

■ Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.

■ Deadline for submission is the end of May.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates (personal and institutional) and frequency of publication, to: Peter Tallack, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK. For further information please telephone Peter Tallack on 071-836-6633 (011-44-71-836-6633 from the United States), extension 2414.

Dispersing the clouds

James F. Crow

The Children of the Atomic Bomb Survivors: A Genetic Study. Edited by James V. Neel and William J. Schull. National Academy Press*: 1991. Pp. 530. \$49.95 (hbk), \$29.95 (pbk).

As soon as the atomic bombs exploded over Hiroshima and Nagasaki, and well before this time for insiders, geneticists were concerned about the genetic effects on the survivors of the bombings. Yet no one had much idea of the possible magnitude of such defects. Most of what was known was based on experiments with the fruitfly *Drosophila*, a dubious basis for extrapolation to man. This concern prompted a large-scale study of the Japanese survivors and their children.

Planning for the project began early in 1946 and the study started in 1948. The Japanese had already begun to look for abnormal births, and from the beginning the research has been done jointly by Japanese and Americans. The original organizing commission, chaired by George Beadle and including H. J. Muller, cautiously noted that the study could well be inconclusive but argued that this unique opportunity should not be lost.

This volume contains reprints of 13 papers, originally published between 1947 and 1991, presenting the results obtained in the genetic study. To these, James Neel and William Schull have added an analytical introduction and an epilogue. A welcome inclusion is the extensive 1956 report, long out of print. The assemblage makes up a hefty tome and gives more details than most readers probably want. Yet the conclusion of the studies can be stated simply: no genetic effects were detected.

The incidence of the various indicators of genetic defects — stillbirths, infant deaths, large congenital malformations, later deaths (up to the age of about 26), cancer, chromosome rearrangements, sex-chromosome aneuploidy, and altered sex ratio and electrophoretically detected proteins — was not significantly changed, either individually or collectively. One reason is that, although some 70,000 children were studied, the radiation dose in the exposed group was not large: the average total dose to both parents was estimated as 0.36 sievert (36 rem to us oldsters). Another reason is that each indicator is affected by many

other factors, so radiation effects could be lost in the noise.

Although the data do not permit any estimate of the lower limit for genetic effects (the confidence limits all include zero), they provide useful upper limits. In one recent paper, Neel and Schull estimate the 'doubling dose' (the amount of radiation required to equal the spontaneous mutation rate per generation) as about 2 Sv for acute radiation and 4 Sv for chronic radiation (background radiation is about 0.001 Sv per year). These numbers are higher than those for mice, but, given the uncertainties, are not inconsistent.

Neel and Schull worry that early exaggerations led survivors to fear for their

dependent and -independent mutations, the doubts about the spontaneous mutation rate in female mice, and the differences between human and mouse oocyte physiology. But regardless of this quibble, the important conclusion, whether one uses mouse or human data, is clear: exposure to low doses of radiation comparable to the background radiation presents very little risk to future generations.

On reading this volume, and especially on rereading the full 1956 report, I was again impressed by the size of the effort and the great difficulties encountered by the US and Japanese scientists, and by the remarkable cooperation of the Japanese subjects. Neel and Schull

IMAGE
UNAVAILABLE
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REASONS

Survivors of the atomic-bomb attack on Hiroshima queueing for treatment.

children, emphasizing as a salutary accomplishment "the dispersal of the ugly clouds which for a period were a serious impediment to the marriage of their children". It might be added that the somatic effects, although statistically real, are small relative to immediate deaths. The total number of excess deaths from cancer so far is a few hundred compared to more than 100,000 immediate deaths.

The last reprinted paper is an attempt to rationalize a seeming discrepancy between the doubling dose in man and mouse. Although the use of the spontaneous mutation rate as a convenient yardstick for describing human risk makes good sense, comparison between mouse and man is less defensible: complications arise from the much shorter life spans of mice, the differences between male and female human mutation rates, the mixture of replication-

emphasize that, contrary to recurring rumours that the US Atomic Energy Commission tried to influence the earlier studies, they were never "aware of any political pressure meant to influence the organization of the study or the way the data were analyzed". Although the book gives a great deal of the history of the project, those eager to learn more will soon be able to read a fuller account recently completed by Schull. I look forward to seeing it in print. □

James F. Crow is in the Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706, USA.

Addendum

Aids in Africa: Its Present and Future Impact by T. Barnett and P. Blakie is published in paperback by Guilford Press in the United States and Canada, price \$18.95. For review, see *Nature* **356**, 393 (1992).

* Distributed by Maruzen Co., Ltd, 3-10 Nihonbashi 2-Chome, Chuo-Ku, Tokyo 103 in Japan, and by Wiley (UK) in Europe, Africa and the Middle East. Price \$60 (hbk), \$36 (pbk).

Motion pictures

Tim J. Mitchison

Cell Movements. By Dennis Bray. *Garland*: 1992. Pp. 406. \$44.95 (hbk); \$27.95 (pbk). (Distributed by European Book Services.)

Molecules of the Cytoskeleton. By Linda Amos and W. Bradshaw Amos. *Macmillan/Guilford Press*: 1991. Pp. 253. £40, \$50 (hbk); £18.99, \$25 (pbk).

THE problems of how cells move, divide and organize their internal contents have been discussed by cell biologists for more than 150 years. This area of study has seen dramatic progress in the past few years with the application of modern molecular techniques to the cytoskeleton, membranes and other parts of the cell. There is a great need for up-to-date textbooks on the subject if we are to recruit young scientists to work on these problems and perhaps finally to solve them.

Dennis Bray's book is based on the format of the highly successful general cell biology textbook *Molecular Biology of the Cell* (Garland, 2nd edn 1989), of which he was one of six authors. The format has proven to be particularly useful for teaching. Each section of Bray's new text is prefaced by a declarative statement and most have an accompanying figure. The header statements are repeated in the table of contents, making it easy to find specific points. The figures are a nice mix of micrographs, diagrams and explanations of experiments, although all are in black and white — addition of colour would have enlivened the pages considerably. The book covers all aspects of cell movement and touches on related issues such as signalling, the extracellular matrix and development. It is probably rather long for an undergraduate course, but is sufficiently detailed to serve as a useful reference source for researchers and interested doctors. In comparison with *Molecular Biology of the Cell*, it goes into more detail on mechanism, groups the material more effectively, and will presumably be much easier to update. The last point is particularly important in such a fast-moving field (no pun intended).

Bray is well qualified to write on cell movement, especially as his own work in the area has been influential, although the book is not unduly influenced by his research interests — cortical flow, for example, rated only a single page in the text. I was interested to read his synthesis of the literature on actin-based cell locomotion, which is well balanced and

explained. But I was left a little unclear as to what he considered to be the important unsolved issues, or indeed what would constitute a solution to the old problem of the mechanism of this type of movement.

All in all though, Bray has produced probably the most comprehensive and up-to-date text on cell motility. It will be very valuable for upper-level graduate teaching, although a comprehensive video supplement would put the book into a class of its own as a teaching aid — it is impossible for even the best text and micrographs to convey the beauty and complexity of different types of cell movement. Video sequences of some of the more important movements would inspire an undergraduate audience and make courses more memorable.

Molecules of the Cytoskeleton by L. Amos and W. B. Amos is pitched at a higher level than Bray's text. It is, as the title implies, unabashedly molecular, starting from the molecules and going on to the biology rather than the other way around. Almost every page has a diagram of a molecule or polymer, often taking up half the page or more. I really enjoyed the way the molecules dominate the text — it is clear that the authors are totally familiar with, and indeed very fond of these proteins. The cut-out and paste-up models of actin filaments and microtubules, for example, are particularly helpful in understanding the anatomy of these polymers. The book will surely be most valuable for graduate students and researchers in the cytoskeleton and related fields, for whom certain parts should be made compulsory reading; but it could also form the basis of a challenging undergraduate course, albeit one that might fire up some young minds.

Where the book does ascend to biological mechanisms, it is concise and thoughtful. The section on actin-based cell locomotion, for example, is well organized and readable, making the key issues a little easier to discern than in Bray's text and thus perhaps more stimulating to a graduate audience. Inevitably, some exciting recent developments are not covered. Kinesin, for example, gets a fairly short section compared with microtubule-associated proteins — the kinesin gene family has burgeoned greatly since the book was written, and it would have been fascinating to learn what the authors make of related kinesins that move in opposite directions on the microtubule lattice. I look forward to finding out in a second edition. □

Tim J. Mitchison is in the Department of Pharmacology, University of California, San Francisco, Box 0450, S-1210, San Francisco, California 94143-0450, USA.

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Time-dependent ozone depletion potentials for short- and long-term forecasts

Susan Solomon & Daniel L. Albritton

Aeronomy Laboratory, National Oceanic and Atmospheric Administration, Boulder, Colorado 80303, USA

Assessments of long-term ozone depletion by CFCs have relied on their relative effects as quantified by ozone depletion potentials (ODPs). These long-term ODPs, based on steady-state atmospheric impacts, are not appropriate for making shorter-term (decade-scale) forecasts. Time-dependent ODPs, derived using an empirical approach, show that some of the hydrochlorofluorocarbons proposed as replacements for CFCs may induce significant ozone destruction in the short term.

INCREASING abundances of man-made halocarbons in the atmosphere have led to extensive ozone depletion and have prompted international regulation of the emissions of many of these compounds. The measure used in international regulation is the ozone depletion potential^{1,2} (ODP), which represents the ozone-depleting impact of emission of one substance relative to another (generally CFCl_3). The first policy debates focused on chronic impacts on the ozone layer and were therefore based on long-term ozone depletion potentials appropriate for evaluating relative ozone losses on a timescale of about a century. Current concerns also include limiting the ozone destruction expected in the next few decades, as atmospheric chlorine and bromine will rise to and decline from the highest values ever experienced on the planet. Relative ozone losses for many species in this key period are larger than those at steady-state, in some cases by a factor of 10 or more. Although compounds with short atmospheric lifetimes tend to have relatively small ozone losses over timescales of several decades or more, they are far more damaging to the ozone layer in the short term (especially if they contain bromine atoms). Simple empirically based methods can provide a conceptual framework for understanding the relationship between short-term and long-term ODPs and accurately evaluating the impact of future halocarbon regulations on the state of the ozone layer for the next decades and centuries.

ODPs and regulation

As part of scientific guidance to public and industrial policy decisions, the stratospheric research community has developed and refined the ODP index^{1,2}. As it is a relative measure, the ODP does not provide information about the absolute amount of ozone destruction but does reveal the trade-offs associated with the use of one molecule rather than another. This subject has become increasingly important as the search for substitutes for the most damaging species has intensified, and concerns about increased near-term ozone losses have grown (for example, because it has been recognized that the coupling of halogen chemistry with background stratospheric and volcanic aerosols³ might enhance ozone depletion; the 1991 eruption of Mount Pinatubo brought this to the foreground). Such indices are useful guides to industry, indicating which of the halocarbon substitutes are likely to be the most environmentally acceptable and hence merit investment in production capability.

The ODPs used in the discussions that led to the Montreal Protocol and regulations were steady-state values, which reflect the integrated ozone depletion achieved at steady state for continuous emissions of a given mass per year of the compound and the reference molecule. This definition is also equivalent to the integrated effect of a pulsed emission. Ozone losses integrated over particular time periods are generally used in

FIG. 1 Time-dependent ozone depletion potentials in polar regions relative to CFC-11 deduced using semi-empirical information and equation (1).

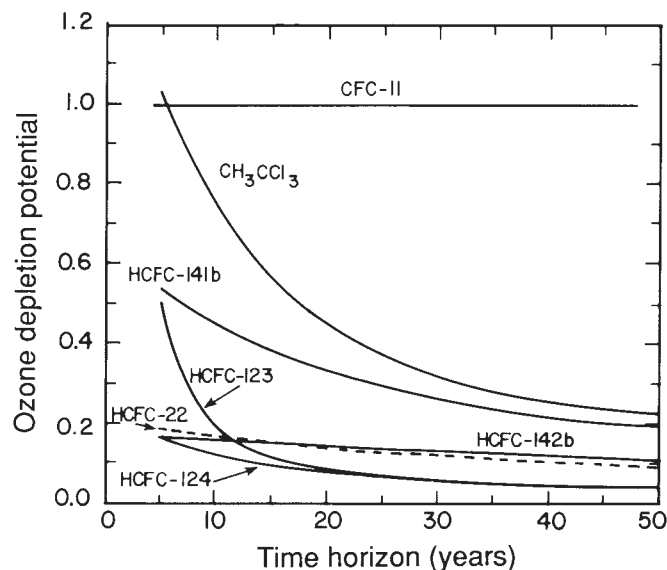


TABLE 1 Relative fractional dissociation, lifetimes and steady-state ODPs of halocarbons

Species	$F_x/F_{\text{CFC-11}}$	Total atmospheric lifetime (yr)*	Stratospheric lifetime (yr)*	Steady-state empirical polar ODP
CFC-11 (CFCl_3)	1.00	55	55	1.0
CFC-113 ($\text{CFCl}_2\text{CF}_2\text{Cl}$)	0.75†	110	110	1.10
CCl_4	1.06†	47	47	1.08
CH_3CCl_3	1.08†	6.1	47	0.12
HCFC-142b ($\text{CH}_3\text{CF}_2\text{Cl}$)	0.36†	22.4	215	0.07
HCFC-22 (CF_2HCl)	0.36†	15.8	240	0.05
HCFC-141b (CH_3CFCl_2)	0.71†	10.8	73	0.11
HCFC-123 (CF_3CHCl_2)	1.112*	1.7	47	0.02
HCFC-124 (CF_3CHFCl)	0.52*	6.9	129	0.02
HCFC-225ca ($\text{CF}_3\text{CF}_2\text{CHCl}_2$)	1.09*	2.8	60	0.02
HCFC-225cb ($\text{CF}_2\text{ClCF}_2\text{CHFCl}$)	0.50*	8.0	120	0.03
CH_3Br	1.08‡	1.5	35	0.57
H-1211 (CF_2ClBr)	1.04‡	19.4	40	4.1
H-1301 (CF_3Br)	0.835‡	67	67	12.5
H-2402 ($\text{CF}_2\text{BrCF}_2\text{Br}$)	0.92‡	25	38	5.9

Values are relative to CFC-11 as the reference gas.

* Ref. 3. † Ref. 10. ‡ Ref. 13.

these indices rather than instantaneous values, at least in part because of the need to represent the effects of cumulative exposure to enhanced ultraviolet light resulting from ozone decreases (for example, human skin cancer). Steady-state indices were appropriate for the policy discussions of the late 1980s for two reasons. First, those deliberations were the very first of such international debates, and the simplest reasonable indices were the most likely to be adopted. Second, as the halocarbons under debate were mainly the chlorofluorocarbons (CFCs), which have lifetimes ranging from several decades to centuries, the steady-state values were appropriate. In short, decisions were being made on the chronic effect of such emissions, namely, on the relative ozone losses that were predicted to occur gradually over the next century.

Both the scientific and the policy situations are now quite different. Specifically, the Antarctic ozone hole has been discovered⁴, and mid-latitude ozone losses are now observed to be occurring in all seasons⁵. Moreover, the research of the past several years has shown that the cause of the Antarctic ozone hole is anthropogenic halocarbons^{2,6}, and the weight of evidence indicates that these compounds are also the cause of a substantial component of the mid-latitude ozone losses. Many of these

findings have led world governments to decide to phase out CFCs and halons and have raised questions about current time-tables (whether the phase-out should be by the turn of the century or faster). As a result of the public awareness of the scientific findings and the current regulations, global production of many halocarbons is beginning to decrease. The 'chronic' (long-term) policy issues thus seem to be largely settled, at least in principle. But the long atmospheric residence times of many of the compounds in question (ranging from decades to centuries), together with expected emissions, imply that the stratospheric levels of total chlorine (hereafter referred to as chlorine loading) will continue to increase from the present value of ~ 3.4 parts per 10^9 by volume (p.p.b.v.), reach a peak of ~ 4.1 p.p.b.v. near the turn of the century and slowly decline thereafter^{3,7}. Although it is accepted that less-ozone-depleting substitutes for the CFCs and halons are required to allow the most damaging compounds to be phased out, questions are being raised as to whether to limit the period of use and the volume of the substitutes (particularly some of the hydrochlorofluorocarbons, HCFCs). Indeed, such substitutes are now sometimes referred to as 'transitional substances'. From these considerations, together with the predicted time profile of chlorine abundances, the policy discussions can be differentiated into two areas of emphasis: the long term, that is, the second half of the next century when chlorine loading could drop low enough for the Antarctic ozone hole to disappear (≤ 2 p.p.b.v.); and the next few decades, from the 1990s through the 2010s, during which the peak halocarbon abundances will occur and a rapid increase in the use of substitute molecules is expected. The policy issues in each domain are rather different and could be aided by rather different ozone-depleting indices, as we will describe.

The long-term domain is fairly well characterized by steady-state ODPs, which should therefore continue to be useful to policy-makers in coming years as they consider how to approach the 'best-case' rate of decrease of chlorine (for example, the 1992 amendments to the Montreal Protocol). But decisions on the relative merits of substitutes to use in the near-term are likely to be based in part on estimates of the relative impacts of these compounds on the ozone layer during the impending transition period (lasting a few decades) of maximum atmospheric chlorine loading. The near-term ODPs (representing the expected relative ozone losses over the next 10–20 years) are quite different from the corresponding steady-state values^{2,8}. To aid the debate, we focus here on providing ODPs over specific time horizons tailored for both the near-term and long-term future of the ozone

FIG. 2 Time-dependent semi-empirical ozone depletion potentials in polar regions relative to CFC-11 for a range of chlorine- and bromine-containing species of varying atmospheric lifetimes.

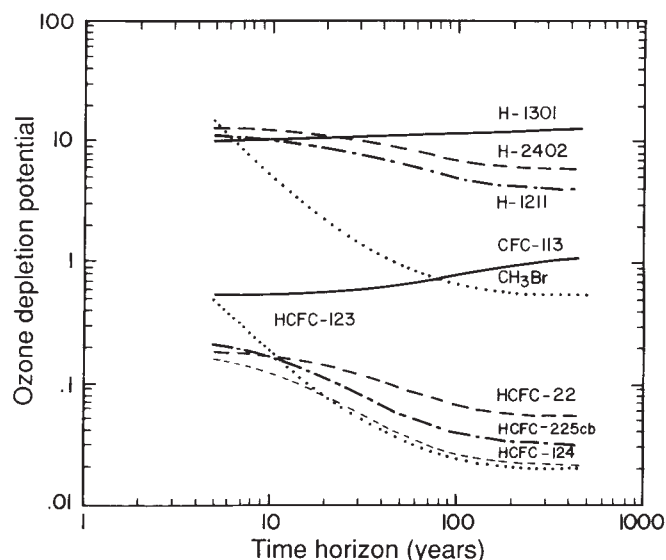


TABLE 2 Semi-empirical polar ozone depletion potentials

Species	Time horizon (years)								
	5	10	15	20	25	30	40	100	500
CFC-113	0.55	0.56	0.58	0.59	0.60	0.62	0.64	0.78	1.09
CCl ₄	1.26	1.25	1.24	1.23	1.22	1.22	1.20	1.14	1.08
CH ₃ CCl ₃	1.03	0.75	0.57	0.45	0.38	0.32	0.26	0.15	0.12
HCFC-142b	0.17	0.16	0.15	0.14	0.13	0.13	0.12	0.08	0.07
HCFC-22	0.19	0.17	0.15	0.14	0.13	0.12	0.10	0.07	0.05
HCFC-141b	0.54	0.45	0.38	0.33	0.30	0.26	0.22	0.13	0.11
HCFC-123	0.51	0.19	0.11	0.08	0.07	0.06	0.04	0.03	0.02
HCFC-124	0.17	0.12	0.10	0.08	0.07	0.06	0.05	0.03	0.02
HCFC-225ca	0.42	0.21	0.14	0.10	0.08	0.07	0.05	0.03	0.02
HCFC-225cb	0.21	0.17	0.14	0.11	0.10	0.09	0.07	0.04	0.03
CH ₃ Br	15.3	5.4	3.1	2.3	1.8	1.5	1.2	0.69	0.57
H-1211	11.3	10.5	9.7	9.0	8.5	8.0	7.1	4.9	4.1
H-1301	10.3	10.4	10.5	10.5	10.6	10.7	10.8	11.5	12.5
H-2402	12.8	12.2	11.6	11.0	10.6	10.1	9.4	7.0	5.9

Reference gas is CFC-11.

layer, using a simple but physically based procedure.

Tools for evaluation of ODPs

Two-dimensional models have been used to estimate globally averaged steady-state ODPs for a broad range of source gases^{2,9}. One-dimensional atmospheric models have been used mainly for the computationally demanding task of deducing time-dependent globally averaged ODPs by examining the detailed ozone response over many decades following a step change in halocarbon release². Model estimates of ODPs have been the subject of increasing scrutiny because of difficulties associated with accurately simulating atmospheric transport phenomena (which control the stratospheric distributions of halocarbons and thereby their release of reactive chlorine or bromine there) and heterogeneous chemical processes known to be critical to ozone destruction, especially in polar regions.

In contrast to these model-based approaches, direct observations of halocarbons together with estimates of atmospheric lifetimes can be used to evaluate local steady-state ODPs for particular locations¹⁰. Stated briefly, measurements of the halocarbons provide direct information on their release of reactive chlorine or bromine, which in turn controls the local ozone loss and hence the local ODP¹⁰. For bromocarbons, the evaluation of the ODP also requires consideration of the greatly enhanced efficiency of bromine for ozone destruction as compared with chlorine. Limited observations indicate that bromine is ~40 times more effective than chlorine for ozone loss¹⁰, a value that will be adopted here. Some molecules for which direct measurements are not available can also be evaluated semi-empirically using correlation relationships with other gases (such as methane) based on model calculations¹⁰. These correlations depend mainly on the ratio of calculated lifetimes¹¹ and are not very sensitive to the aforementioned modelling problems. This empirical approach can be used to check how accurately models can simulate ozone loss and halogen release, gives a clearer conceptual understanding of the differences in ODPs among various compounds, and can be done simply, quickly and cheaply for any molecule.

Following this approach, the semi-empirical time-dependent ODP at any point in the stratosphere can be derived simply from the following equation, in which CFC-11 is chosen as the reference gas

$$\text{ODP}_x(t) = \left\{ \frac{F_x}{F_{\text{CFC-11}}} \right\} \frac{M_{\text{CFC-11}}}{M_x} \frac{n_x}{3} \alpha \times \frac{\int_{t_s}^t \exp(-(t-t_s)/\tau_x) dt}{\int_{t_s}^t \exp(-(t-t_s)/\tau_{\text{CFC-11}}) dt} \quad (1)$$

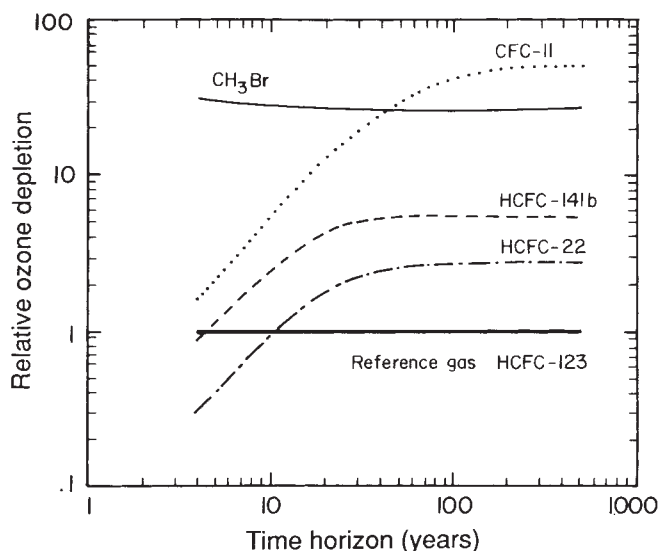
The term in brackets $\{F_x/F_{\text{CFC-11}}\}$ denotes the fraction of the halocarbon species x injected into the stratosphere that has been

dissociated compared with that of CFC-11 (both fractions obtained from measurements). M_x , $M_{\text{CFC-11}}$, τ_x and $\tau_{\text{CFC-11}}$ indicate the molecular weights and atmospheric lifetimes of species x and CFC-11, respectively, and n_x is the number of chlorine or bromine atoms in the molecule (note that CFC-11 contains three chlorine atoms per molecule). α is a factor needed when species x is a bromocarbon and reflects the higher efficiency of bromine atoms for ozone loss as compared with chlorine atoms. t_s is the time required for a molecule to be transported from the surface to the region of the stratosphere in question, and t is time. Table 1 presents values of $\{F_x/F_{\text{CFC-11}}\}$ for several halocarbons, deduced from source gas measurements in the Arctic lower stratosphere (~16–20 km altitude), together with current estimates of the stratospheric and overall atmospheric lifetimes of these species and their steady-state ODPs. Uncertainties in ODPs for chlorinated species are dominated at present by uncertainties in atmospheric lifetimes. Large uncertainties in estimated atmospheric OH concentrations dominate the absolute uncertainty in the lifetimes of the HCFCs, rendering the absolute values of their ODPs uncertain by perhaps as much as a factor of two³. Note, however, that the relative values of the lifetimes and hence the relative ODPs of the HCFCs depend not on the absolute OH abundance, but on relative rate constants for reaction with OH measured in the laboratory, which are believed to be accurate to within ~10–30%. For the bromine-containing compounds, uncertainties in lifetimes are significant, as well as in the value of α . The present approach can be readily updated as improved estimates of these quantities become available.

Tracer studies indicate that the time required for transport of air from the tropopause to the polar lower stratosphere is ~3–5 years^{13,14}, implying a time lag between the release of a halocarbon and the onset of ozone depletion (so that the ODP cannot be defined for shorter times). The time horizons for the ODP estimates presented here are those for a 4-year lag time. Different lag times would change the time when a given ODP occurs but not its value. This information, together with equation (1) and the information in Table 1, allows us to evaluate the time-dependent ODPs for various halocarbons in the polar lower stratosphere (Fig. 1). The results are extremely similar to those of published one-dimensional model calculations (Fig. 4.3-11 of ref. 2), indicating that the simple formula captures the primary physical processes controlling the time-dependent response of the model.

Large ozone losses have been observed in polar regions and their links to halogen chemistry have been established^{2,3,6}, making the ODPs appropriate to polar conditions a subject of particular importance in policy considerations. Nevertheless, the correspondence between polar lower-stratospheric and globally averaged ODPs is an important factor in interpreting the numbers presented here. The relationship between polar

FIG. 3 Time-dependent semi-empirical ozone depletion potentials in polar regions relative to HCFC-123 for a selected set of compounds.



lower-stratospheric and globally averaged ODPs depends on (1) the magnitude of the ozone losses there compared to the global total and (2) whether or not the local ODPs at other latitudes (for example at mid-latitudes, if these contribute significantly to the global mean) differ greatly from the polar values. Observations have established that lower-stratospheric ozone depletions dictate the total column losses^{2,3} at all latitudes and thus the global average. Polar regions dominate the global ozone loss, and therefore the globally averaged ODP, because of the very large losses occurring there¹⁰. Furthermore, detailed study of global ozone and tracer measurements shows that the mid-latitude local ODPs in the lower stratosphere are very close to those of polar regions (within ~10%) for HCFCs¹⁰, so that the ODPs presented here are expected to be close to the globally averaged ODP for the HCFCs. Uncertainties in the latitudinal variations in α make the extrapolation to the global scale more problematic for bromine-containing species, but these are likely to be within a factor of two of the polar values³.

ODPs over various time horizons

Table 2 and Fig. 2 present time-dependent ODPs for a number of important chlorine- and bromine-containing compounds. The time-dependent ODPs for the 500-year time horizon are nearly equal to the steady-state values for all of the gases considered (Table 1). For species with atmospheric lifetimes comparable to CFC-11, the ODP varies little with time (examples are CCl_4 ; H-1301). For compounds with overall lifetimes longer than CFC-11 (such as CFC-113), smaller ODPs are obtained over short timescales, but these grow over time. For species with short overall lifetimes (for example, the HCFCs, CH_3CCl_3 , CH_3Br , H-1211, H-2402), the ODPs over short timescales can be much larger than the long-term values.

Let us consider the simultaneous release of one kilogram each of HCFC-123, CFC-113 and CFC-11. All of these compounds are rapidly mixed throughout the troposphere. HCFC-123 is destroyed within the troposphere on a timescale of ~1.7 years, whereas CFC-113 and CFC-11 are destroyed only on entering the stratosphere. Nonetheless, a fraction of the released amounts of all three compounds enters the stratosphere rapidly (within the first few weeks following release, before significant loss has taken place). Once in the stratosphere, the ozone destruction depends on the release of reactive chlorine by each species (and hence on the stratospheric lifetime of the source gas compared to that of CFC-11, reflected in the value of $\{F_x/F_{\text{CFC-11}}\}$). The ozone destruction induced by the emitted HCFC-123 in the first few years is large, nearly half that of CFC-11 and close to that of CFC-113 (Table 2). Over longer time horizons, the ODP for HCFC-123 quickly diminishes as it is destroyed, whereas that

for CFC-113 increases, because of their respective total atmospheric lifetimes. Put differently, every molecule of HCFC-123 releases its chlorine rapidly while in the stratosphere and is thus a potent destroyer of ozone, but fewer and fewer of them are able to do so over time (because of its very short total atmospheric lifetime). Essentially all of the injected HCFC-123 is destroyed in less than a decade. The ODP for HCFC-123 nonetheless requires more than a century to approach its steady-state value because of the slow breakdown of CFC-11, so that the reference gas requires about a century to 'catch up' to HCFC-123. This comparison illustrates the difference between the integrated and instantaneous relative ozone losses caused by molecules with differing lifetimes.

HCFC-123 and HCFC-124 have nearly the same long-term ODP, but the short-term ODP of HCFC-123 is larger than that of HCFC-124 because of its considerably shorter stratospheric lifetime and hence its higher value of $\{F_x/F_{\text{CFC-11}}\}$. Although a short lifetime does imply a quick atmospheric recovery should a problem with a compound be recognized and its emissions halted, this comparison shows that the shortest atmospheric lifetime does not necessarily imply minimal impact upon the ozone layer. The value of $\{F_x/F_{\text{CFC-11}}\}$ dominates the ODP over the shortest timescales (immediately after release) whereas the lifetime generally dominates the longer-term ODP and determines how it changes with time horizon. It is worth noting that evaluations of steady-state or time-dependent chlorine loading potentials^{2,7,8} are based, for simplicity, on the assumption that all chlorine injected into the stratosphere is equivalent in terms of ozone destruction and so do not incorporate the effects of $\{F_x/F_{\text{CFC-11}}\}$ discussed here.

Bromocarbons tend to have relatively short stratospheric lifetimes (because of the weak chemical bonding of bromine) and therefore have $\{F_x/F_{\text{CFC-11}}\}$ values near 1. The near-term ODP of a bromocarbon then depends on the value of α , the number of bromine atoms in the molecule and its molecular weight. The 5-year ODPs for all of the bromine-containing molecules considered here are estimated to be ~10–15. A value of α of 20 (80) would change these to 5–7.5 (20–30). Note that this includes the relatively short-lived species, CH_3Br ; all of the bromine-containing species are potent near-term ozone destroyers. Only over longer timescales does the short lifetime of CH_3Br cause its time-dependent ODP to decrease.

Policy implications

Clearly, the most damaging chlorinated compounds tend to be those with a large number of chlorine atoms and a long total atmospheric lifetime (such as CCl_4 , CFC-113). The ideal substitute would be one with a short overall atmospheric lifetime

determined by rapid tropospheric removal, but a very long stratospheric lifetime (to limit $\{F_x/F_{\text{CFC-11}}\}$). Unfortunately, the stratospheric and total atmospheric lifetimes are coupled because the same chemical breakdown processes occur to some extent in both the troposphere and stratosphere (photolysis, reaction with OH and so on). Some of the substitute molecules, particularly those destroyed uniquely by reaction with OH (such as HCFC-22, HCFC-142b) have fairly long stratospheric lifetimes, and therefore smaller values of $\{F_x/F_{\text{CFC-11}}\}$ and relatively small near-term ODPs, but somewhat larger long-term ODPs. Use of such compounds rather than, for example, HCFC-123 would imply trade-offs between near-term and longer-term ozone damage.

As the ODP over any time horizon represents the total amount of ozone loss resulting from emission of that molecule relative to that of an equal amount of CFC-11, the time-dependent ODPs can be used to evaluate the impact of different emission scenarios (see Table 2). For example, if HCFC-141b were continuously emitted at the present rate of CFC-11 release (in kilograms) and emissions of the latter were immediately eliminated, the total ozone destroyed in the next 10 years or so would be about half that which would be obtained if CFC-11 emission were continued. Within a century, however, the ozone loss for HCFC-141b at this emission rate would be ~13% of that obtained for an equal rate of CFC-11 emission. A similar case for HCFC-123 results in ozone losses for the next 10 years that are ~20% of those obtained for CFC-11 release, whereas a century of emission yields ~2% of the ozone loss.

Given the certain phase-out of CFC-11 and the fact that multiple substitutes are being used in some applications, it may be useful to consider ODPs using a different reference molecule to evaluate the relative effects of possible substitutes. Note that such ODPs could be derived from Table 2 for any chosen reference molecule simply by division. For the purposes of illustration, Fig. 3 presents time-dependent ODPs for a few gases with HCFC-123 as the reference gas. These data reinforce the fact that HCFC-141b and CH_3Br have some of the largest relative impacts on the ozone layer in both the long and short-term among molecules not yet scheduled to be phased out under the London amendments to the Montreal protocol.

Table 2 and Fig. 3 clearly illustrate that the rapid phase-out of CFCs, CH_3CCl_3 , CCl_4 and halons is the main factor in reducing ozone destruction over both short and long time horizons. To the extent that they are needed, substitute compounds can aid that goal and so help to protect the ozone layer.

But the next 20 years are likely to encompass the highest atmospheric halocarbon abundances that the Earth will (presumably) experience. If policy makers were to decide to phase out compounds with large steady-state ODPs as soon as this is practical because of their chronic effects (such as the Antarctic ozone hole), and to place a time limit on the widespread use of substitutes for the same reason, then they would probably consider how best to minimize the growing ozone losses that will occur during this transition. Another goal may be to minimize the risks of further 'surprises' produced by the nonlinear chemistry of unprecedentedly large halocarbon abundances or its coupling to unpredictable influences such as volcanic eruptions. The absolute ozone destruction depends on the atmospheric processes involved and may change character beyond certain thresholds of reactive chlorine content (as in the appearance of the Antarctic ozone hole, or the possible onset of similar behaviour in Arctic regions in the future). Therefore, the critical issues involve identifying which substitutes are needed, for how long, and in what quantities.

Short-term ODPs for the currently uncontrolled compounds could be a principal factor in those decisions. In applications for which multiple substitutes exist (such as HCFC-141b and HCFC-123), industry may choose to invest in the compounds with the smaller short-term ODPs (Fig. 3). Similarly, governments may consider placing appropriately low ceilings on the production of compounds with larger projected ODPs in the next few decades, and higher ceilings on those with smaller short-term ODPs. It may, for example, seem prudent to allow emissions only of those compounds whose ODPs (relative to CFC-11) over the next 10 years or so lie below 0.2 (for example HCFC-124b, HCFC-22, HCFC-123, HCFC-124, HCFC-225ca and HCFC-22cb), if these can meet world needs and if those needs cannot reasonably be fulfilled by non-chlorinated species. A few substitutes offer short-term advantages but longer-term disadvantages (for example, HCFC-22 or HCFC-142b relative to HCFC-123), so that the net impact over a range of timescales should be considered when designing control strategies. A mix of compounds with allowed release rates and termination dates could be formulated with the goal of minimizing both short-term and long-term ozone losses; additional important factors to be considered would include the feasibility, the economic and technological aspects, and the impact of the emitted compounds on greenhouse warming.

The time-dependent ODPs presented here are offered as an aid to those decisions, many of which are already at hand or will be faced in the coming year. □

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The complete DNA sequence of yeast chromosome III

S. G. Oliver¹, Q. J. M. van der Aart², M. L. Agostoni-Carbone³, M. Aigle⁴, L. Alberghina⁵, D. Alexandraki⁶, G. Antoine⁷, R. Anwar¹, J. P. G. Ballesta⁸, P. Benit⁷, G. Berben⁹, E. Bergantino¹⁰, N. Biteau⁴, P. A. Bolle⁹, M. Bolotin-Fukuhara¹¹, A. Brown¹, A. J. P. Brown¹², J. M. Buhler¹³, C. Carcano³, G. Carignani¹⁰, H. Cederberg¹⁴, R. Chanet⁷, R. Contreras¹⁵, M. Crouzet⁴, B. Daignan-Fornier¹¹, E. Defoor¹⁶, M. Delgado¹⁷, J. Demolder¹⁵, C. Doira¹¹, E. Dubois¹⁸, B. Dujon¹⁹, A. Dusterhoff²⁰, G. Erdmann²⁰, M. Esteban¹⁷, F. Fabre⁷, C. Fairhead¹⁹, G. Faye⁷, H. Feldmann²¹, W. Fiers¹⁵, M. C. Francinques-Gaillard¹¹, L. Franco⁸, L. Frontali²², H. Fukuhara⁷, L. J. Fuller²³, P. Galland⁶, M. E. Gent¹, D. Gigot¹⁸, V. Giliquet⁹, N. Giansdorff¹⁸, A. Goffeau^{24,27}, M. Grenson²⁵, P. Grisanti²², L. A. Grivell²⁶, M. de Haan²⁸, M. Haasemann²⁷, D. Hatat²⁸, J. Hoenicka⁸, J. Hegemann²⁰, C. J. Herbert²⁹, F. Hager⁹, S. Hohmann¹⁴, C. P. Hollenberg³⁰, K. Huse¹⁴, F. Iborra¹¹, K. J. Indge¹, K. Isono³¹, C. Jacq²⁸, M. Jacquet¹¹, C. M. James¹, J. C. Jauniaux²⁵, Y. Jia²⁹, A. Jimenez⁸, A. Kelly³², U. Kleinhaus³⁰, P. Kreisl²⁷, G. Lanfranchi¹⁰, C. Lewis²³, C. G. van der Linden³³, G. Lucchini³, K. Lutzenkirchen³⁰, M. J. Maat²⁶, L. Mallet¹¹, G. Mannhaupt²¹, E. Martegani⁵, A. Mathieu⁷, C. T. C. Maurer³³, D. McConnell³², R. A. McKee²³, F. Messenguy¹⁸, H. W. Mewes²⁷, F. Molemans¹⁵, M. A. Montague³², M. Muzi Falconi³, L. Navas¹⁷, C. S. Newlon³⁴, D. Noone³², C. Pallier¹¹, L. Panzeri³, B. M. Pearson²³, J. Perea²⁸, P. Philippson²⁰, A. Pierard¹⁸, R. J. Planta³³, P. Plevani³, B. Poetsch²⁷, F. Pohl³⁵, B. Purnelle²⁴, M. Ramezani Rad³⁰, S. W. Rasmussen³⁶, A. Raynal¹¹, M. Remacha⁸, P. Richterich³⁵, A. B. Roberts¹², F. Rodriguez⁵, E. Sanz⁸, I. Schaaff-Gerstenschlager¹⁴, B. Scherens¹⁸, B. Schweitzer²⁰, Y. Shu²⁸, J. Skala²⁴, P. P. Slonimski²⁹, F. Sor⁷, C. Soustelle¹¹, R. Spiegelberg²⁰, L. I. Stateva¹, H. Y. Steensma², S. Steiner²⁰, A. Thierry¹⁹, G. Thireos⁶, M. Tzermia⁶, L. A. Urrestarazu²⁵, G. Valle¹⁰, I. Vetter²¹, J. C. van Vliet-Reedijk³³, M. Voet¹⁶, G. Volckaert¹⁶, P. Vreken³³, H. Wang³², J. R. Warming¹, D. von Wettstein³⁶, B. L. Wickstead¹², C. Wilson²², H. Wurst³⁵, G. Xu³⁰, A. Yoshikawa³¹, F. K. Zimmermann¹⁴ & J. G. Sgouros²⁷

The entire DNA sequence of chromosome III of the yeast *Saccharomyces cerevisiae* has been determined. This is the first complete sequence analysis of an entire chromosome from any organism. The 315-kilobase sequence reveals 182 open reading frames for proteins longer than 100 amino acids, of which 37 correspond to known genes and 29 more show some similarity to sequences in databases. Of 55 new open reading frames analysed by gene disruption, three are essential genes; of 42 non-essential genes that were tested, 14 show some discernible effect on phenotype and the remaining 28 have no overt function.

THE sequence of the DNA molecule of chromosome III from the budding yeast *S. cerevisiae* has been completed by a consortium of 35 European laboratories within the framework of the European Community's Biotechnology Action Programme. The sequence is 315 kilobases long and contains 182 open reading-frames (ORFs) encoding putative proteins of ≥ 100 amino acids. So far, 55 novel ORFs have been subjected to functional analysis by gene disruption. In addition to the putative protein-encoding genes there are 10 transfer RNA genes, of which four had previously been defined by suppressor mutations. Regions of sequence variation between chromosomes III of different strains of *S. cerevisiae* have been identified, as have the differences between the physical and genetic maps. These data indicate that systematic genome sequencing projects can reveal new functions that have been missed by more traditional approaches and also illuminate the mechanisms of genome evolution. Most importantly, they reveal that our knowledge of molecular genetics is far from complete and that we are ignorant about the biological function of the majority of genes in a eukaryotic genome as small and well-defined as that of yeast.

The bakers' yeast *S. cerevisiae* is one of the most important experimental organisms for studying eukaryotic molecular genetics¹. This yeast has a very small nuclear genome which, at about 14 megabases (Mb), is less than four times the size of that of the bacterium *Escherichia coli*. Like all eukaryotes, yeast

divides its nuclear genome between a number of linear chromosomes. The 16 yeast chromosomes have been defined by both genetic² and physical³ analyses. Each contains a single duplex DNA molecule⁴ whose average size is similar to that of the genome of a T-even bacteriophage and thus represents an achievable objective for nucleotide sequencing with current technology⁵. Such a sequence analysis would not only advance yeast molecular genetics but also provide information important to our understanding of the genomes of higher eukaryotes. The pattern of gene expression in yeast is essentially similar to that in higher organisms¹. Moreover, a number of yeast genes have been identified that share both structural and functional homology with genes of multicellular eukaryotes.

Despite their small size, yeast chromosomes resemble their counterparts from higher organisms in structure and in their mechanisms of replication, recombination and segregation⁶. The ease with which the yeast genome can be manipulated by both classical and recombinant DNA techniques has enabled all the functional chromosome components to be isolated: centromeres⁷, telomeres⁸ and replication origins⁹. These advances in our knowledge of chromosome structure and function, gained by the use of gene cloning techniques, have been paralleled by the study of chromosome recombination using a combination of classical and molecular approaches¹⁰. The availability of the complete nucleotide sequence of a yeast chromosome permits

¹Manchester Biotechnology Centre, UMIST, Manchester M60 1QD, UK; ²Department of Cell Biology Genetics, Leiden University, The Netherlands; ³Dipartimento di Genetica e di Biologia dei Microrganismi, Università di Milano, Italy; ⁴LBMS, F-33000 Bordeaux, France; ⁵Dipartimento di Fisiologia e Biochimica, Università di Milano, Italy; ⁶Instituto Molecular Biology and Biotechnology, PO Box 1527, GR-71110 Heraklio, Crete; ⁷Institut Curie, Centre Universitaire, F-91405 Orsay, France; ⁸Centro de Biología Molecular, E-28049 Madrid, Spain; ⁹Faculté des Sciences Agronomiques, B-5030 Gembloux, Belgium; ¹⁰Dipartimento di Chimica Biologica, I-35100 Padova, Italy; ¹¹Institut de Génétique et Microbiologie, Université de Paris-Sud, 91405 Orsay, France; ¹²Department of Molecular and Cell Biology, University of Aberdeen, UK; ¹³Service de Biochimie CEN Saclay, F-91191, France; ¹⁴Institut für Mikrobiologie, D-6100 Darmstadt, Germany; ¹⁵Lab. voor Moleculaire Biologie, B-9000 Gent, Belgium; ¹⁶Katholieke Universiteit Leuven, voor Geneeskunde, B-3001 Leuven, Belgium; ¹⁷La Cruz del Campo S.A., PO Box 53, E-41080 Sevilla, Spain; ¹⁸Research Institute of CERIA-COOVI, B-1070 Bruxelles, Belgium; ¹⁹Institut Pasteur, F-75724 Paris Cedex 15, France; ²⁰Institut für Mikrobiologie und Molekularbiologie der Universität, 6300 Giessen, Germany; ²¹Institut für Physiologische Chemie, Universität München, 8000

München, Germany; ²²University of Rome, Department of Cell and Developmental Biology, I-00185 Roma, Italy; ²³AFRC Institute of Food Research, Norwich Research Park, Colney NR4 7UA, UK; ²⁴Unité de Biochimie Physiologique, Université de Louvain, B-1348 Louvain-la-Neuve, Belgium; ²⁵Université Libre de Bruxelles, Lab. Cell Physiology and Yeast Genetics, B-1050 Bruxelles, Belgium; ²⁶University of Amsterdam, Section for Molecular Biology, NL-1098 SM Amsterdam, The Netherlands; ²⁷Martinsried Inst. for Protein Sequences, D-8033 Martinsried, Germany; ²⁸Génétique Moléculaire, École Normale Supérieure, F-75005 Paris, France; ²⁹Centre de Génétique Moléculaire du CNRS, F-91190 Gif-sur-Yvette, France; ³⁰Institut für Mikrobiologie der Universität Düsseldorf, D-4000 Düsseldorf 1, Germany; ³¹Department of Biology, Kobe University, Kobe 657, Japan; ³²Trinity College, Department of Genetics, Dublin 2, Ireland; ³³Department of Biochemistry and Molecular Biology, Vrije Universiteit, NL-1081 HV Amsterdam, The Netherlands; ³⁴Department of Microbiology and Molecular Genetics, New Jersey Medical School, NJ07103, USA; ³⁵Fakultät für Biologie der Universität, D-7750 Konstanz, Germany; ³⁶Carlsberg Lab., DK-2500 Copenhagen Valby, Denmark; ³⁷Commission of the European Communities, B-1049 Brussels, Belgium

a detailed comparison of genetic and physical maps and so helps to define local variations in the frequency of recombination¹¹. *S. cerevisiae* also contains a number of transposons (called Ty elements) which show similarities to retroviruses and other mobile genetic elements found in multicellular eukaryotes¹² and which are a major source of chromosome polymorphisms.

All of this suggests that sequencing the yeast genome should provide a useful paradigm to guide our interpretation of the information gained from the sequences of larger genomes when these become available. Indeed, at 14 Mb (compared with 3,360 Mb for the human genome), the 16 chromosomes of yeast might be regarded as comprising a basic gene-set required for the maintenance of a eukaryotic mode of cell organization and propagation. There are also practical reasons why the determination of the yeast genome sequence should be a useful precursor to larger enterprises such as sequencing the genomes of higher plants or humans. First, the functional analysis of novel genes discovered from the sequence is facilitated by the easy methods for gene disruption and replacement¹³ which are available in yeast. Second, high-capacity cloning vectors (yeast artificial chromosomes, or YACs¹⁴), propagated in yeast, are important for creating clone banks of human or plant genes. Thus, it is useful to establish in the first instance the genome sequence of the vehicle organism. Finally, sequencing the yeast genome can act as a pilot scheme for assessing new techniques designed to speed sequence determination and analysis¹⁵. The yeast chromosome III sequence reported here has been determined mainly by conventional techniques. In fact, only 25 kb of the 385-kb sequence from which the final 315-kb consensus was derived has been obtained using automated techniques (7%). The following approaches were used to divide the primary clones (indicated by upper bars in Fig. 1) into fragments of a size suitable for sequencing: directed subcloning (19 kb; 5%), shotgun cloning (82 kb; 21%), chromosome walking with synthetic oligonucleotides (137 kb; 36%), and nested deletions (147 kb; 38%).

Chromosome III was divided between the laboratories of the Consortium and accredited clones were distributed to each group. Each laboratory was responsible for one or two work units (1 work unit is 8 kb of primary sequencing and 3 kb of overlap sequencing) and for disruptions of novel ORFs (some disruptions were performed instead of re-checking overlaps). The individual laboratories forwarded their data to the Martinsried Institute for Protein Sequences (MIPS), where the sequence was assembled by means of the GCG package¹⁶ and a variety of algorithms were used to analyse ORFs and other sequence elements. MIPS also assembled a database for all extant *S. cerevisiae* DNA sequences, scanning not only the computer databases but also all published results. The project was initiated in January 1989 and the sequence completed by May 1991. Sequencing of entire eukaryotic genomes will probably require worldwide collaboration and our chromosome III sequence project represents an organizational and technological model for such enterprises.

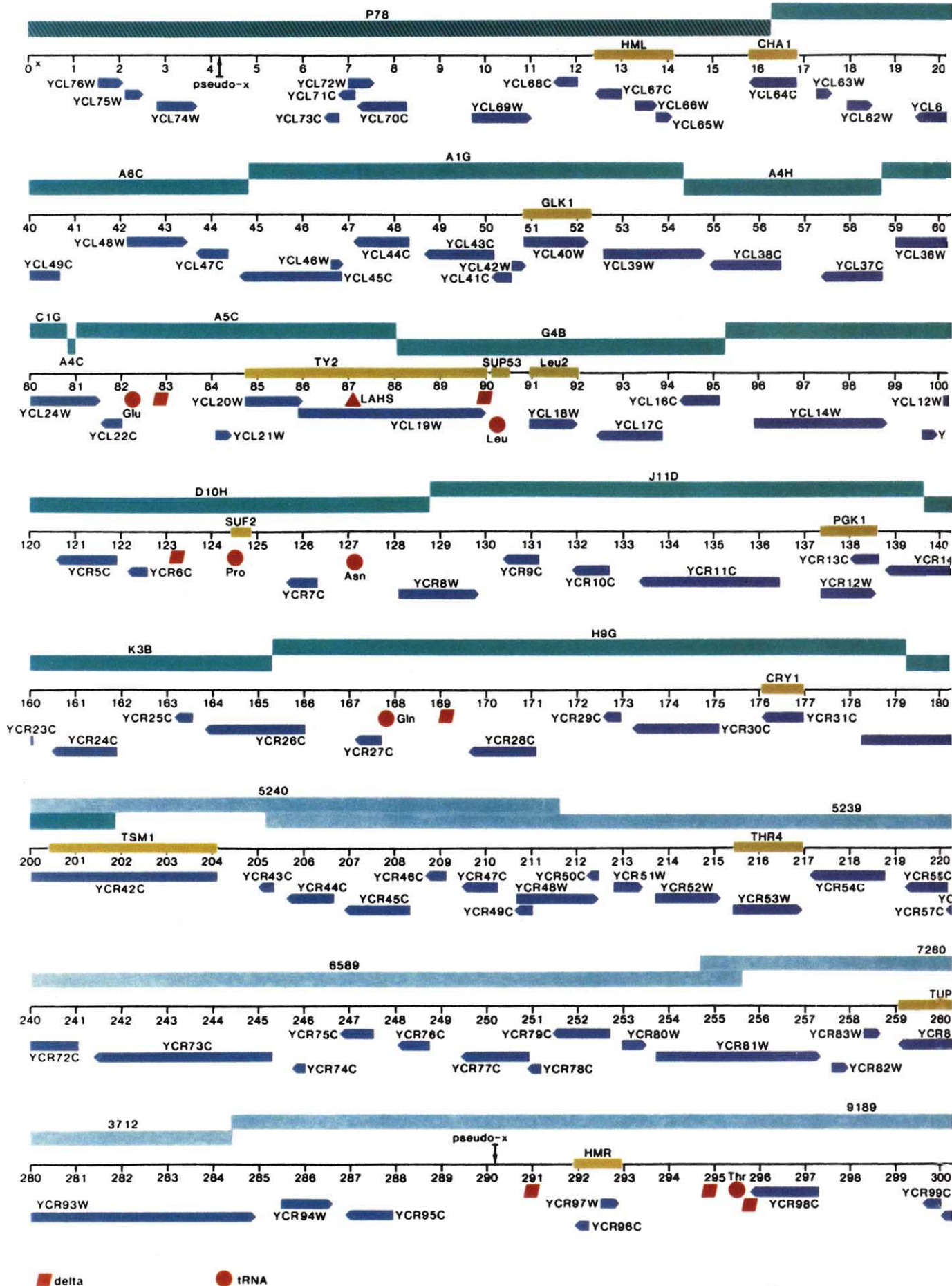
Chromosome III

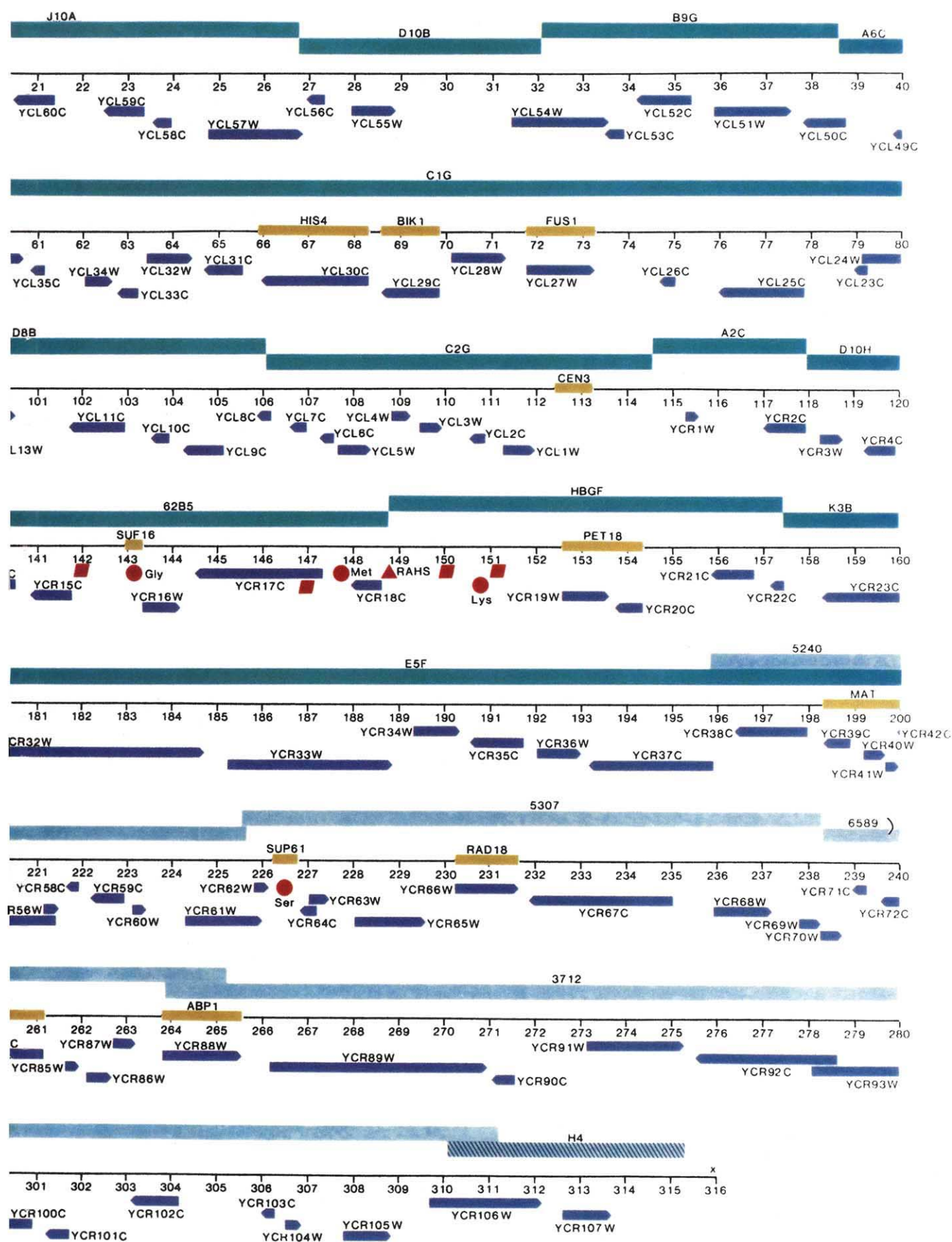
Chromosome III is the third smallest chromosome in *S. cerevisiae*; its size was estimated from pulsed-field gel electrophoresis studies to be 300–360 kb¹⁷. This chromosome has been the subject of intensive study, not least because it contains the three genetic loci involved in mating-type control: *MAT*, *HML* and *HMR*¹⁸. *MAT*, in the middle of the right arm of the chromosome, is the expression locus that determines mating-type, whereas *HML* and *HMR* (located near the left and right ends of the chromosome) represent silent repositories of mating-type information. These three loci share nucleotide sequence homology, which means that intrachromosomal recombination events may generate circular derivatives of the chromosome. An *HML* × *MAT* recombination produces a 63-μm ('small ring') derivative which has been isolated¹⁸ and used to generate an ordered clone bank of the chromosome in the shuttle vector

YIp5 (ref. 19). This bank, necessarily, lacks a small part of the left arm and about half of the right arm of the chromosome; the latter was available from a series of clones constructed in lambda and cosmid vectors²⁰. These two gene banks were used to generate the sequence covering 280 kb of the 315 kb of chromosome III and were derived from *S. cerevisiae* strains XJ24-24a^{18,19} and AB972 (ref. 21). For the remaining 35 kb, clones derived from strains A364A¹⁹ and DC5 (refs 22 and 23) were used. These strains are closely related (XJ24-24a, A364A) or isogenic (AB972, DC5) to the standard yeast laboratory strain 3288C (ref. 24). The data are summarized in this article and a full listing of the sequence is available in the EMBL Data Library (accession number X59720) and will also be the subject of a more extensive publication.

A comparison of the chromosomes III from these strains, as well as from other laboratory and industrial isolates, permits an assessment of the degree of sequence polymorphism between different representatives of the same chromosome. Ty insertions in the chromosome are mainly confined to two regions that have been dubbed the left-arm and right-arm transposition hot spots (LAHS and RAHS, respectively^{25–28}). Many strains (but not XJ24-24a) contain another Ty, distal to the RAHS, close to the gene *CRY1*. Interactions between this distal Ty and the RAHS seem to be a major cause of chromosome length polymorphisms. Some strains have a deletion in this region which causes a number of phenotypic effects, including respiratory deficiency²³, whereas other strains (including the progenitor S288C) have this region duplicated (ref. 19, and B.L.W. *et al.*, manuscript in preparation). We have sequenced a large overlap between the Newlon and Olson clone banks and this has provided detailed information about this polymorphic region as well as permitting the elucidation of the authentic unique sequence of the chromosome. The version of the chromosome presented in Fig. 1 is 315,357 base pairs (bp) long; it starts at the left telomere²⁹ and ends within the X sequence of the right telomere, about 400 bp from the terminus. A single Ty2 element is shown in the LAHS,

FIG. 1 Location of ORFs and known genetic markers on chromosome III. Royal blue arrow-bars: ORFs (≥100 amino acids) are designated as follows: Y (for yeast) C (the third letter of the alphabet for the third chromosome); L or R (for the left and right arm of the chromosome); a number that designates the relative position of the ORF on its arm of the chromosome (the most centromere-proximal ORF being given the number 1); w or c (for Watson or Crick strand) to indicate the orientation of the ORF. All ORFs shown start with an ATG codon. Number scale is in kb. Upper blue bars indicate the clone used to obtain the primary sequence of that region of the chromosome. Dark turquoise bars: clones J10A [36, 6], D10B [16], B9G [23], A6C [25], A1G [18], A4H [17], C1G [30, 20], A4C [20, 1], A5C [1], G4B [1], D8B [1], C2G [1], A2C [2], D10H [4], J11D [24], 62B5-2D [28], HBGF [21], K3B [9], H9G [35, 14] and E5F [29, 19] were from the YIp5 bank of the small ring form of the chromosome (numbers in square brackets indicate the laboratories responsible for sequencing that clone, see addresses); all, except 62B5-2D (from DC021/DC022#62) and HBGF (from CN31c), were from strain XJ24-24a (ref. 19). The *Bam*HI junctions between these clones were checked using a λCh4A bank derived from strain A364A (ref. 19). Light turquoise bars: clones 7121 [12, 32], 3270 [5, 3], 5240 [11, 13], 5239 [26, 33], 5307 [7, 15], 6589 [8], 7260 [21, 20], 3712 [22, 10] and 9189 [33, 26, 7] were from strain AB972 (ref. 20). Clones 7121 and 3270 are in the overlap region between the Newlon¹⁹ and Olson²⁰ banks and are not indicated in the figure. The ends of the chromosome were sequenced using clones (dark turquoise, cross-hatched) p78 [36, 2, 11, 19] derived from strain A364A (ref. 19) and (light turquoise, cross-hatched) H4 [25] from strain DC5 (ref. 22). The Sanger dideoxy-sequencing technique⁴⁹ was used for the entire chromosome with the exception of parts of clone D10B, for which the chemical procedure of Maxam and Gilbert⁵⁰ was employed. Full details of the sequencing strategy will be published (S.G.O. *et al.*, manuscript in preparation) and the full 315,356-bp sequence has been deposited in the EMBL Nucleotide Sequence Data Library under the accession number X59720. Two ORFs from the *pet18* complex are <100 amino acids in length and so are not shown in the figure. The following ORFs represent newly discovered essential genes: YCL17c, YCL14c, YCR69w.





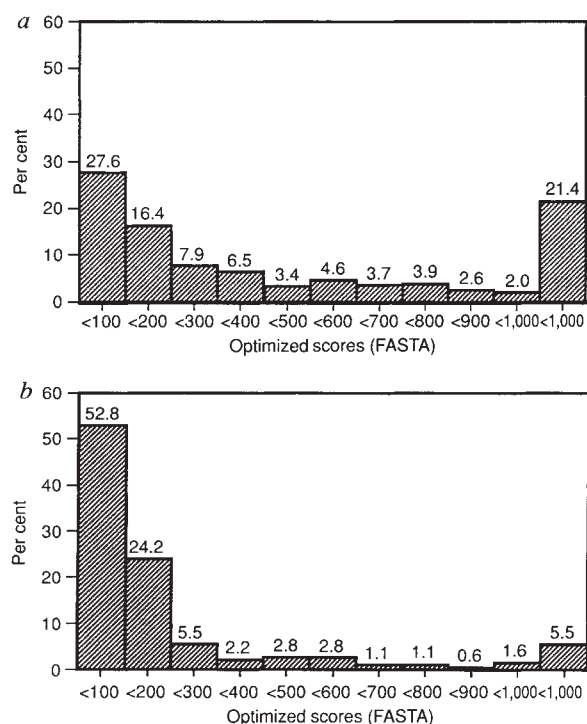


FIG. 2 Distribution of FASTA⁵¹ scores for all yeast proteins (a) and for the chromosome III ORFs (b). The amino-acid sequence of the 968 protein-encoding yeast genes present in the data bases and the 182 ORFs in chromosome III were subjected to a similarity search against all known protein sequences present in the MIPSX data base using the Pearson and Lipman⁵¹ FASTA algorithm. (MIPSX is a merged database comprising PIR-International, SwissProt and automatic translations of both the EMBL and GenBank nucleotide-sequence data sets.) Optimized scores were used to compile the data. In this analysis, scores due to matches with identical sequences were omitted and the next highest score to a non-identical sequence was taken.

but no transposon is shown in the RAHS; this is because the fused tandem pair of Ty elements found at this site in S288C, AB972, DC5 and XJ24-24a has been produced by a recombination event that deleted part of the unique sequence of the chromosome, including an ORF and a tRNA gene²⁷. The form of the chromosome shown, and the sequence entered in the EMBL Data Library, permits the inclusion of these genes. The following lengths of the chromosomes III in the source strains have been calculated from the sequence data: XJ24-24a, 324 kb; A364A, 322 kb; DC5, 336 kb; S288C and AB972, 345 kb.

Open reading frames

The transcript analysis of Yoshikawa and Isono²² predicted that chromosome III specifies 160 messenger RNA molecules (≥ 350 nucleotides) in addition to the transcripts of the Ty elements. This is likely to be an underestimate as the northern blots were run only on poly(A)⁺ RNA extracted from cells growing in a rich medium. Nonetheless, the agreement between the transcript map and the ORF map is, in general, very high. For example, Yoshikawa and Isono²² show two transcripts of 6.5 and 4.2 kb in the region corresponding to base pairs 178,250 to 188,900 in the sequence. These transcript sizes agree very well with those of the two ORFs in this region, YCR32w (6,501 bp) and YCR33w (3,678 bp)^{30,31}. The complete sequence analysis of the chromosome reveals 182 ORFs of ≥ 100 amino acids. ORFs of this length have $<0.2\%$ probability of occurring by chance in *S. cerevisiae* DNA³². All 182 ORFs begin with an ATG; those ORFs whose sequence is entirely contained within another reading-frame have been excluded from the analysis. Seventeen ORFs overlap one another by between 7 and 449 bp; these are all included. Figure 1 shows the distribution of the 182 ORFs in the chromosome sequence. Of the 182 putative protein-encoding genes shown, only 34 appear on the *S. cerevisiae* genetic map (ref. 2, and R. K. Mortimer, personal communication). These numbers illustrate the fact that, even in a genome as small and as intensively studied as that of yeast, only a minor fraction of the genes has been identified by classical means. Therefore one justification for large-scale sequencing projects is the identification of new areas of investigation, as demonstrated by our sequence analysis of a single small eukaryotic chromosome.

The MIPS YEASTPROT database currently contains the sequence of 968 protein-encoding genes from *S. cerevisiae*. Of these 968, a total of 251 (26%) show amino-acid sequence similarity to other proteins encoded by the same species, giving FASTA scores of ≥ 200 . A further 286 (30%) show a similar level of homology to proteins from organisms other than *S. cerevisiae*. A survey of the 145 newly discovered ORFs from chromosome III presents a strikingly different picture. Only $\sim 10\%$ (15/145) show significant similarity (FASTA ≥ 200) to non-*S. cerevisiae* genes already in the databases, 15 more are homologous with other genes from *S. cerevisiae* itself, and 117 (80%) show no significant homology to any previously sequenced genes. (Figure 2 shows the distribution of FASTA scores for the set of 182 chromosome III ORFs and all previously known ORFs from *S. cerevisiae*.)

The 15 newly discovered chromosome III gene products that show significant similarity to non-*S. cerevisiae* proteins are listed in Table 1a. Some, for example alcohol dehydrogenase and asparagyl-tRNA synthetase, are routine housekeeping proteins, but many others are unexpected. For instance, one of the highest scores is given by YCL17c, which is homologous to the *nifS* gene product of nitrogen-fixing bacteria^{33,34}. *S. cerevisiae* does not fix atmospheric nitrogen³⁵, yet this highly conserved gene is essential to its growth³⁶. Recent evidence suggests that this gene is involved in both tRNA processing and mitochondrial metabolism (P. Leong-Morgenthaler *et al.*, manuscript submitted). YCL74w shows a high degree of similarity to a *copia*-like protein from *Arabidopsis*³⁷, the tobacco transposon (Tnt1; ref. 38) and to *copia*³⁹ itself. Chromosome III contains representatives of all four classes of yeast transposons²⁵⁻²⁸; YCL74w may indicate the presence of a fifth class. If the set of 15 proteins encoded by the rest of the *S. cerevisiae* genome that show greatest similarity to proteins from other species is examined, then the majority of matches are to proteins from other yeasts and filamentous fungi and all 15 are proteins of well-known function. These findings demonstrate that genome sequencing reveals, at a high frequency, new functions that have not been discovered by classical techniques and suggest that yeast molecular geneticists are working on only a small subset of the problems presented by their experimental organism.

The set of chromosome III ORFs that show significant similar-

ity to previously characterized genes on other yeast chromosomes (Table 1b) reveals no particular pattern, except that kinase genes are overrepresented (5/15; YCL24w, YCR8w, YCR91w, YCR73c, YCR38c). This group includes similarities to some genes, like *YKR*, which were expected to have homologues elsewhere in the genome⁴⁰. It may be necessary to perform several inactivations to reveal the role of genes of this type, but the task of assigning functions to novel genes identified by systematic sequencing is still more readily addressed in yeast than in any other eukaryote. Of the 145 novel ORFs found in the chromosome III sequences, 55 (38%) have been subjected to gene disruption¹³. For three genes this was a lethal event. Of the remainder, 42 disruptants have been tested for other effects on phenotype, using a standard battery of tests compiled by P.P.S., and in 14 cases some effect was observed. The phenotypes included heat and cold sensitivity, respiratory deficiency (or an enhanced level of mutation to that phenotype), hypersensitivity to various drugs, sterility and defects in budding, sporulation or meiotic recombination. This leaves 28 of 45 genes tested (62%) for which no phenotype can be assigned; Goebl and Petes⁴¹ had previously suggested, based on a study of random disruptions, that 70% of the yeast genome was 'dispensable'. It is unlikely that these genes make no contribution to the fitness of the organism and this implies that our understanding of yeast physiology and cell biology is lagging behind our molecular genetic analysis.

A very small proportion of genes sequenced from *S. cerevisiae* contain introns (about 2%; ref. 42). On this basis, it might be expected that chromosome III would include three intron-containing genes. Apart from the mating-type loci¹⁸, three have been detected (YCR31c and two others that are less than 100 amino acids long) by searching for the TACTAAC box and the 5' and 3' splice junctions⁴². One of these genes, *cry1* (YCR31c), was already known; it encodes the ribosomal protein rps59 (ref. 43). A large proportion of intron-containing genes in yeast encode ribosomal protein subunits⁴², but neither the protein-encoding sequences nor the upstream regions of the two small intron-containing genes on the chromosome give any indication that they are ribosomal proteins as well.

Transfer RNA genes

Like most organisms, *S. cerevisiae* contains multiple gene copies encoding iso-accepting species of tRNA. Feldmann⁴⁴ has esti-

mated that yeast contains 360 tRNA genes. Four such genes had been identified as suppressors and mapped to chromosome III (*SUP53*, *SUP61*, *SUF2* and *SUP16*; ref. 2, and R. K. Mortimer, personal communication). The sequence identifies six more (encoding tRNA^{Asn}, tRNA^{Glu}, tRNA^{Gln}, tRNA^{Lys}, tRNA^{Met} and tRNA^{Thr}). A total of seven tRNA genes would be expected on a statistical basis; all of the 10 found are within 500 bp of a Ty or delta element. The frequent association of tRNA genes with such elements has been noted previously^{45,46} and their possible role in the amplification and spread of such genes has been discussed²⁷.

Comparison of genetic and physical maps

The average ratio between genetic and physical map distances in yeast is estimated to be 0.34 centimorgans (cM) per kb (ref. 2). Smaller chromosomes, such as I and VI, have a higher ratio and Kaback *et al.* suggested that this is necessary to ensure at least one crossover occurs at each meiosis so that these small chromosomes can segregate correctly⁴⁷. Within chromosomes, recombination frequency varies widely and both recombination hot spots and cold spots have been identified¹¹. The sequence of chromosome III may suggest some molecular basis for these local variations in recombination frequency. A detailed comparison of the physical map of the chromosome (based on the sequence) and the current version of the genetic map (ref. 2; and R. K. Mortimer, personal communication) is unrealistic as the latter is, necessarily, a compromise between the results of several laboratories and also takes advantage of the emerging physical data. Nevertheless, some instructive generalizations may be made. The average ratio of genetic map distance to physical distance for the chromosome is 0.51 cM per kb; this is in line with the estimates for chromosomes I and VI (0.62 and 0.55 cM per kb, respectively) and supports Kaback's⁴⁷ postulate. The variation in the cM per kb ratio for different intervals along the chromosome is at least 10-fold; it is lowest close to the centromere and greatest midway down each arm. There is thus an approximate correlation between the pattern of genetic recombination and that of transcription along the chromosome²². An association between recombination and transcription has been suggested previously⁴⁸, and it may be that both processes require relatively naked DNA within the chromatin. A region where the frequency of recombination is particularly high is the interval between *MAT* and *thr4* (1.12–1.27 cM per kb); this

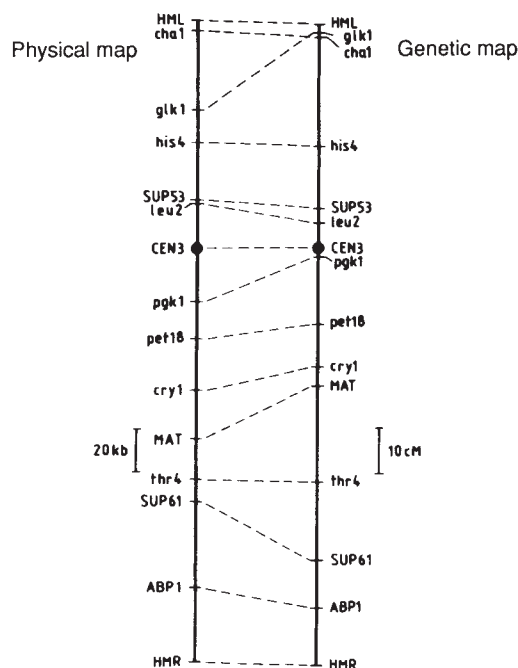


FIG. 3 Comparison of genetic and physical maps. The physical map has been derived from the sequence by assigning each locus to a point in the centre of the appropriate ORF, group of ORFs, or DNA element. Thus each locus, given as a point on this physical map, may represent a region of the chromosome from 0.1 kb to 6 kb in length. The genetic map is derived mainly from the analysis of meiotic recombination events²; fine structure mapping data (permitting estimation of genetic length) are available for only a few genes.

TABLE 1 Chromosome III gene products showing significant homology to extant protein sequences

ORF	Coordinates	Rational gene name	Similar sequence (database: accession no.)	FASTD score	Effect of gene disruption
(a) Newly discovered ORFs showing similarity to non- <i>S. cerevisiae</i> proteins					
YCL57w	24,752-26,887		Rat soluble metalloendopeptidase (Genbank: M61142)	1,012	
YCR92c	278,640-275,500		Human DUC-1 protein upstream of DHFR (EMBL: J04810)	960	Non-lethal, no detectable phenotype*
YCL17c	93,881-92,391	<i>NFS1</i>	<i>Anabaena nifs</i> nitrogen-fixation protein (EMBL: J05111; M21840)	778	Lethal†
YCR2c	117,951-116,986		Mouse DIFF6 protein, regulated by gp90(MEL-14) (EMBL: X13303)	660	
YCR24c	161,932-160,457		<i>E. coli</i> asp-tRNA synthetase (EMBL: M33145)	550	
YCL74w	2,816-3,739		<i>Arabidopsis copia</i> -like protein (EMBL: M53975)	550	
YCR11c	136,474-133,328	<i>ADP1</i>	<i>Drosophila</i> white pigment protein (EMBL: X51749)	512	Non-lethal, no detectable phenotype‡
YCR63w	227,040-227,510		<i>Xenopus</i> G10 protein, developmentally regulated (EMBL: X15243)	480	Slow growth§
YCL43c	50,196-48,631	<i>PDI1</i>	Protein disulphide isomerase precursor (PIR: JX0182; EMBL: M62815, X52313)	2,501	Lethal
YCR105w	307,800-308,882		<i>Zymomonas</i> alcohol dehydrogenase I (EMBL: M32100)	366	
YCR27c	167,715-167,089		<i>Dictyostelium</i> ras-related protein (EMBL: X54291)	309	
YCR65w	228,031-229,626		<i>Drosophila fkh</i> homeotic gene (EMBL: J03177)	286	
YCR57c	221,442-220,126		Human transducin β -2 chain, GTP-binding (Gen Bank: M36429)	266	
YCL11c	102,964-101,684		<i>Xenopus</i> poly(A)-binding protein (PIR: S12000)	227	
YCR107w	312,620-313,708		Tobacco auxin-induced protein (EMBL: X56269)	218	
(b) Newly discovered ORFs showing significant similarity to other <i>S. cerevisiae</i> proteins					
YCL25c	77,880-75,982		General amino-acid permease, GAP1 (EMBL: X52633)	1,527	
YCL64c	16,869-15,790		L-serine dehydratase, SDH1 (EMBL: X52657)	984	
YCL48w	42,140-43,528		Sporulation-specific SPS2 protein (EMBL: M13629)	941	
YCR67c	235,046-231,852		Sec12p membrane glycoprotein (Gen Bank: X13161)	889	
YCL24w	79,119-81,566		Sucrose non-fermenting SNF1 protein kinase (EMBL: M13971)	650	
YCR8w	128,072-129,880		Nitrogen permease reactivator, NPR1 (EMBL: X56084)	445	
YCR52w	213,725-215,173		Gene complementing petite type mutation (EMBL: X62430)	434	
YCR45c	208,342-206,870		Proteinase B precursor (EMBL: M18097)	429	
YCR91w	273,138-275,315		Protein kinase, YKR (EMBL: M24929)	397	
YCL35c	61,142-60,813		Glutaredoxin (PIR: A35492)	396	
YCR73c	245,320-241,379		Protein kinase, Ssp31 (PIR: JQ1118)	321	
YCR83w	258,312-258,692		Thioredoxin II (EMBL: M59169)	272	
YCR28c	171,115-169,580		Allantoate permease (EMBL: M24098)	255	
YCR89w	266,168-270,994		A-agglutinin core subunit, AGA1 (EMBL: M28164)	250	
YCR38c	197,967-196,354		Cell division cycle, CDC25 protein (EMBL: M15458)	201	

Full details of similarity search procedures are given in the legend to Fig. 2. For each ORF, the highest optimum FASTA score found is listed. References to the authors of the pre-existing sequence may be found in each database entry, the accession numbers for which are given in the figure. The term 'Yeast' here indicates the conspecific group, *S. cerevisiae*, *S. carlsbergensis* and *S. uvarum*. For the division between *S. cerevisiae* and non-*S. cerevisiae* matches (see text), first priority was given to similarities to other *S. cerevisiae* sequences; thus, if a match with a FASTA score ≥ 200 with another *S. cerevisiae* sequence was found, this was counted even if a match to a non-*S. cerevisiae* sequence produced a higher score.

TABLE 1—continued

ORF	Coordinates	Rational gene name	Similar sequence (database: accession no.)	FASTD score	Effect of gene disruption
(c) Genes previously sequenced but not assigned to chromosome III					
YCR75c	247,550–246,771	<i>ERS1</i>	ER defect suppressor, ERS1 protein (EMBL: X52468)	1,455	
YCR5c	121,931–120,552	<i>CIT2</i>	Cytosolic citrate synthase (EMBL: Z11113)	2,241	
(d) Genes previously sequenced and assigned to chromosome III but not mapped					
YCL50c	38,775–37,813	<i>DTP1</i>	Diadenosine tetraphosphate phosphorylase, DTP1 (EMBL: M35204)	1,602	
(e) Genes previously mapped on chromosome III but not sequenced					
YCR53w	215,428–216,969	<i>THR4</i>	<i>Corynebacterium</i> threonine synthase (Gen Bank: X56037)	759	
YCR9c	131,144–130,350	<i>RVS161</i>	Reduced viability on starvation, RVS161 protein (PIR: Y01077)	1,280	
(f) Genes previously sequenced and mapped on chromosome III					
YCL18w	90,935–92,026	<i>LEU2</i>	β -isopropyl-malate dehydrogenase (EMBL: X03840)	1,688	
YCL27w	71,768–73,303	<i>FUS1</i>	Cell fusion protein, Fus 1 (EMBL: M16717)	2,425	
YCL29c	68,568–69,867	<i>BIK1</i>	Nuclear fusion protein, Bik1 (EMBL: M16717)	1,304	
YCL30c	68,299–65,903	<i>HIS4</i>	Histidinol dehydrogenase (EMBL: V01310)	3,716	
YCL40w	50,810–52,309	<i>GLK1</i>	Glucokinase (EMBL: M24077)	2,422	
YCL66w	13,271–13,795	<i>HML</i>	Hml α 1 protein	896	
YCL67c	13,007–12,378		Hml α 2 protein (EMBL: V01315)	979	
YCR12w	137,347–138,594	<i>PGK1</i>	Phosphoglycerate kinase (EMBL: J01342)	1,911	
YCR19w	152,544–153,632	<i>PET18</i>	Maintenance of killer, MAK32 protein (PIR: 507695)	1,807	
YCRC20c	154,366–153,722	<i>PET18</i>	MAK31 protein (PIR: 507695)	1,155	
YCR31c	176,968–176,128	<i>CRY1</i>	Ribosomal protein s59 (EMBL: M16126)	617	
YCR40w	199,173–199,697	<i>MAT</i>	Mat α 1 protein	896	
YCR39c	198,909–198,280		Mat α 2 protein (EMBL: V01315)	979	
YCR42c	204,128–199,908	<i>TSM1</i>	Temperature-sensitive lethal TSM1 protein (EMBL: M60486)	6,911	
YCR66w	230,224–231,684	<i>RAD18</i>	DNA repair protein, Rad18 (EMBL: X125880)	2,315	
YCR84c	261,186–259,048	<i>TUP1</i>	Repressor protein (EMBL: M35861)	3,156	
YCR88w	263,802–265,577	<i>ABP1</i>	Actin-binding (EMBL: X51780)	2,674	
YCR97w	292,568–293,051	<i>HMR</i>	Mat α 1 protein	896	
YCR96c	292,271–291,915		Mat α 2 protein (EMBL: V01315)	979	

* Region between *Cla*I site at 278,001 to the *Xba*I site at 277,004 replaced with *URA3*.

† Gene disruption experiments for this ORF are detailed in ref. 36, and in P. Leong-Morgenthaler *et al.*, manuscript submitted.

‡ *URA3* inserted into *Bgl*II site at 134,370.

§ *URA3* inserted into *Xba*I site at 227,218.

|| Region between *Bst*EII site at 48,846 to the *Bst*EII site at 50,887 replaced with *URA3*. The techniques used for the other 51 gene disruptions may be summarized as: 23 insertion mutations (9, *URA3*; 6, *HIS3*; 5, MiniMu; 2, *TRP1*; 1, *HIS4*) and 28 deletion/replacement mutations (15, *URA3*; 6, *HIS3*; 5, *LEU2*; 1, *TRP1*; 1, *LYS2*).

partly explains the apparent paucity of markers in this segment of the genetic map. There is only a single discrepancy in the gene order derived from the sequence and that in the genetic map; the order of *glk1* and *chal*, on the far left arm of the chromosome, is reversed. These data are summarized in Fig. 3.

The sequence data also provide evidence of past recombination events. A 245-bp sequence between residues 290,656 and 290,901 on the right arm (Fig. 1) shows a high degree of homology with the X element found in all yeast telomeres²⁹. A similar sequence, 252 bp long, is found between residues 4,065 and 4,317 at the left end of the chromosome. It may be that chromosome III was once even shorter than it is now and that it has grown by recombination events involving its telomeres. Both the instability of linear plasmids and small artificial chromosomes⁶ in yeast, as well as the Kaback rule⁴⁷, suggest that there may be selection against short chromosomes in *S. cerevisiae*. Moreover, a region near the middle of the left arm of chromosome XI has been shown by our analysis to contain sequences homologous to both the X and Y' telomere elements (B.D. *et al.*, unpublished results).

Discussion

In sequencing the entire unique 315 kb of yeast chromosome III, we have generated 385 kb of total sequence. Thus 22% of the chromosome has been independently sequenced by at least two laboratories. The level of agreement between these sequence determinations was very high, the mismatch frequency being 0.4 per kb for clones from the same strain and 6.2 per kb for clones from different strains. In the latter case, 65% of the mismatches in ORFs occur in the third base of the codons. A re-check of the mismatch regions by the different pairs of laboratories reduced the discrepancies to zero, where clones came from the same strain, but had no significant impact on

the number of differences between strains. In an independent check of the sequence, the restriction map predicted from the sequence has been compared with that derived by experiment (I. Collins and C.S.N., unpublished results). Of 504 6-bp restriction sites sampled, only four showed a discrepancy between the predicted and experimental maps (a putative error rate of 1.3 per kb). All four discrepancies represent predicted sites that could not be identified by experiment, therefore these sites may not be recognized by the appropriate restriction enzyme owing to context effects or DNA modifications. The chromosome III sequence is the largest data set available that has been subjected to such independent checking and so indicates the level of accuracy that larger genome projects may achieve using current technology.

The chromosome III sequence has revealed 145 novel protein-encoding genes and a start has been made on their functional analysis. The results so far indicate that there are vast areas of yeast genetics of which we are completely ignorant and emphasize the need for molecular genetics and physiological studies to proceed hand-in-hand. The data also call for a radical re-appraisal of our view of the yeast genetic map. The availability of the sequence establishes unequivocally the locations of the different genes on the chromosome. In consequence, the genetic map acquires a different emphasis; it becomes much more a tool with which to study recombination and the dynamics of chromosome evolution. The goal of sequencing the entire yeast genome is achievable with present technology and this sequence will prove at least as important to the future development of eukaryotic molecular biology as the classical *S. cerevisiae* genetic map has in the past. The complete sequence of the yeast genome will open up new areas of molecular genetics and establish a foundation for the interpretation of sequence data from higher organisms. □

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A protein of the endoplasmic reticulum involved early in polypeptide translocation

Dirk Görlich, Enno Hartmann, Siegfried Prehn[†] & Tom A. Rapoport[‡]

Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, O-1115 Berlin-Buch, Germany

[†] Institute of Biochemistry, Humboldt University, Hessische Strasse 3-4, O-1040 Berlin, Germany

[‡] To whom correspondence should be addressed

To identify components of the mammalian endoplasmic reticulum involved in the translocation of secretory proteins, crosslinking and reconstitution methods were combined. A multispanning abundant membrane glycoprotein was found which is in proximity to nascent chains early in translocation. In reconstituted proteoliposomes, this protein is stimulatory or required for the translocation of secretory proteins.

THE translocation of most secretory proteins across the mammalian endoplasmic reticulum (ER) membrane is initiated in the cytoplasm by binding of the signal sequence of the nascent chain to the signal recognition particle through its polypeptide subunit of relative molecular mass 54,000 (M_r 54K)¹⁻³. Upon interaction of the signal recognition particle with its membrane receptor (docking protein), the nascent chain is transferred into the membrane⁴⁻⁶. The mechanism and components of the subsequent translocation process are unknown. None of the proteins so far implicated (for example, signal peptidase complex, ribophorins, mp30, signal sequence receptor complex; for review, see ref. 7) has been shown to be essential. In the yeast *Saccharomyces cerevisiae*, three membrane proteins (Sec61p, 62p and 63p) are required for translocation^{8,9}. No homologues of these proteins have yet been detected in mammals.

To study the molecular environment of polypeptides passing through the membrane, crosslinking methods have been used. A glycosylated protein of M_r 35K (refs 10-12) or 39K (refs 13, 14) from canine microsomes gave major crosslinks with nascent

secretory and membrane proteins after their transfer from the signal recognition particle into the membrane. On the basis of the properties of the crosslinking partner, a 34K glycoprotein was purified and termed the signal sequence receptor (now called SSR α)^{10,15,16}. It is a protein that spans the membrane once and is part of a heterotetrameric membrane protein complex (ref. 17, and our unpublished results), but is probably not involved in signal sequence recognition¹³. Nevertheless, circumstantial evidence suggests that this SSR complex is in the translocation site (for review, see ref. 7).

We found that the extent of crosslinking of short nascent chains is low with SSR α . To establish whether another glycoprotein could be responsible for the high degree of crosslinking during the early stages of translocation, we tested proteoliposomes reconstituted from a detergent extract with a defined composition of glycoproteins for the appearance of crosslinked products. A single protein, later termed TRAM (for translocating chain-associating membrane protein), turned out to be responsible and was purified. The sequence of TRAM, as

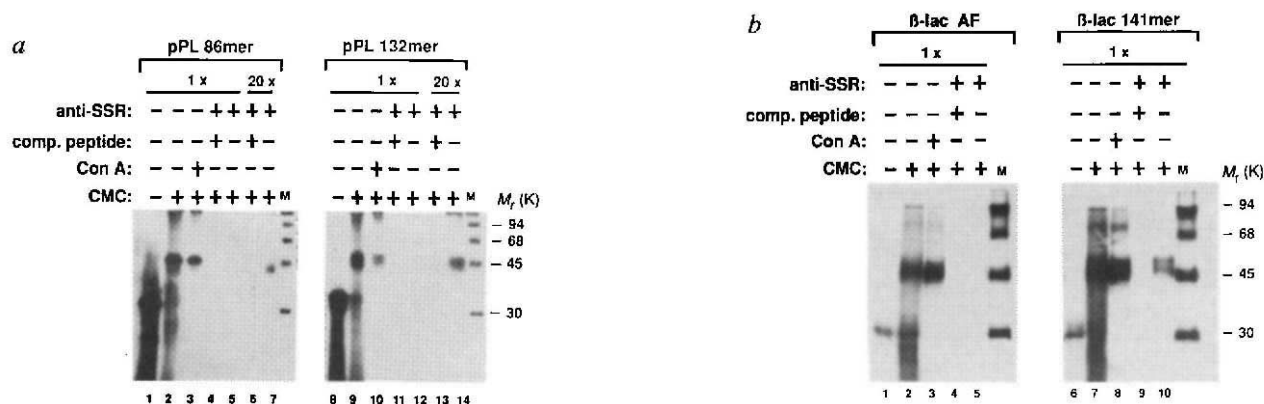


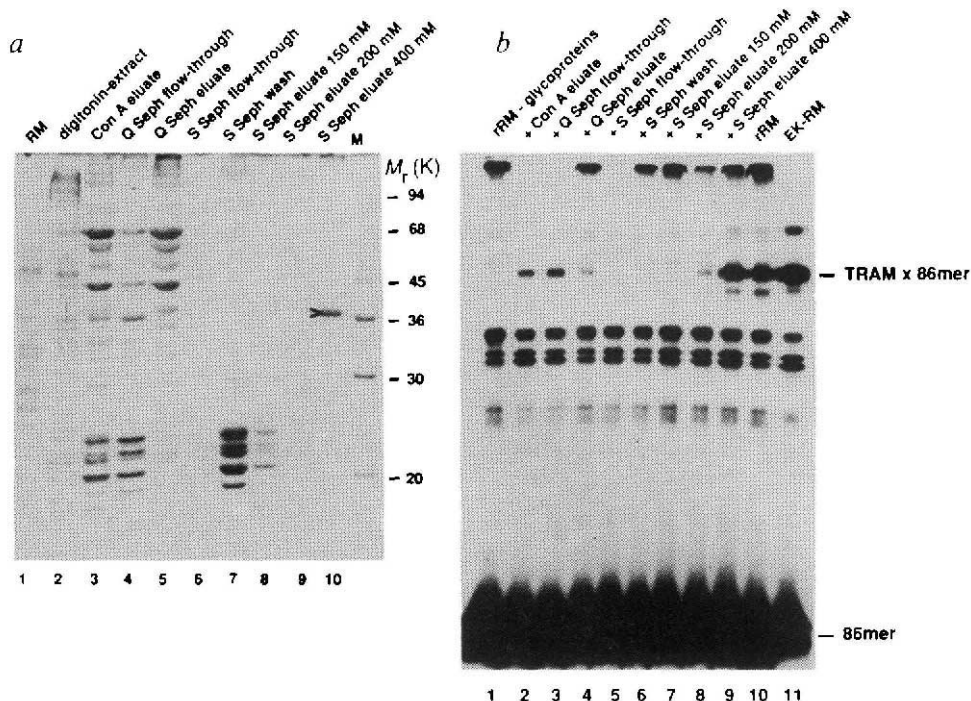
FIG. 1 Crosslinking of SSR α to nascent chains. **a**, Radiolabelled preprolactin (pPL) chains of 86 or 132 amino acids (pPL 86mer and pPL 132mer respectively) were synthesized *in vitro* by translation of truncated messenger RNAs in the presence of canine pancreas microsomes. After crosslinking with the reagent 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-4-toluene sulphonate (CMC), products were bound to concanavalin A-Sepharose (Con A) or immunoprecipitated with antibodies against SSR α (anti-SSR). The specificity of immunoprecipitation was tested by adding excess peptide (comp. peptide) against which the antibodies were raised. Where indicated, 20 times more material was used for immunoprecipitation (20 $\times$). **b**, Crosslinking with pre- β -lactamase chains of about 70 (β -lac AF) or 141 (β -lac 141mer) amino acids, respectively. The doublets of the crosslinks with the longer fragments probably represent products with different linkages between the molecules. Lanes

M, M_r standards (K).

METHODS. Truncated mRNAs coding for pPL 86mer, pPL 132mer and β -lac 141mer were produced by *in vitro* transcription of plasmids cut with restriction enzymes. They were translated in the wheat-germ system in the presence of ³⁵S-methionine and high-salt-washed canine microsomes¹⁷. β -lac AF chains were synthesized by translation of β -lac 141mer mRNA for 5 min in the presence of 50 nM signal recognition particle from canine pancreas before addition of 1 mM cycloheximide and microsomes¹⁷. Crosslinking with CMC and immunoprecipitation with affinity-purified antibodies directed against a peptide corresponding to the C-terminal 12 amino acids of SSR α have been described¹⁷. For competition, 50 μ g ml⁻¹ peptide was added. Binding to con A was as before¹⁷, except with 1% Tween 20 instead of NP40 detergent in the con A-binding buffer.

FIG. 2 Purification of TRAM. *a*, Protein pattern at different stages of purification (Coomassie blue staining after SDS-polyacrylamide gel electrophoresis). RM, rough microsomes; Q Seph, Q-Sepharose; S Seph, S-Sepharose. The arrowhead indicates the position of purified TRAM. About 23 ng TRAM per equivalent (eq.) RM (for definition, see ref. 30) were obtained, corresponding to an overall yield of about 50%. Lane M, M_r markers. *b*, Crosslinked products produced by CMC with the 86mer of preprolactin and reconstituted proteoliposomes containing different fractions of glycoproteins (shown in part *a*) (fluorogram after SDS-PAGE). rRM, proteoliposomes reconstituted from an unfractionated cholate extract; rRM-glycoproteins, proteoliposomes reconstituted from a glycoprotein-depleted extract; EK-RM, rough microsomes washed with EDTA and high salt.

METHODS. Purification procedure: RM (60,000 equivalents) were pre-extracted for 30 min on ice with 120 ml 50 mM HEPES/KOH, pH 7.6, 0.4% digitonin, $2 \mu\text{g ml}^{-1}$ leupeptin, 2 mM dithiothreitol (DTT) and subsequently solubilized in 70 ml of 50 mM HEPES/KOH, pH 7.6, 500 mM potassium acetate, 1.5% digitonin, 2 mM DTT, 10% (w/v) glycerol. The extract was cycled twice over a 6-ml con A-Sepharose column. Glycoproteins were eluted overnight at room temperature with 100 ml 1 M α -methylmannoside, 50 mM HEPES/KOH, pH 7.6, 1% digitonin, 2 mM DTT and collected on ice. They were passed over a 15-ml Q-Sepharose Fast-Flow (Pharmacia) column equilibrated in 50 mM HEPES/KOH, pH 7.6, 10% glycerol, 0.5% digitonin, 2 mM DTT. Elution was with 1 M potassium acetate. The flow-through fraction was diluted 1:1 with 50 mM MES/KOH, pH 6.0, and applied to a 1 ml S-Sepharose Fast-Flow (Pharmacia) column. After washing with 50 mM HEPES/KOH, pH 7.6, 100 mM potassium acetate, 0.5% digitonin, step-elution was done by increasing the salt concentration (5 ml each). Fractions to be tested for crosslinking or



protein pattern were precipitated with 13% (w/v) polyethylene glycol (PEG) 6000. Crosslinking assay: A cholate extract was prepared from canine microsomes as described¹⁸, except that 15% (w/v) glycerol instead of sucrose was used in the solubilization buffer. 1 ml cholate extract was depleted of glycoproteins by incubation with 200 μl prewashed con A-Sepharose overnight in the cold. For replenishment, PEG-precipitated glycoproteins were resuspended in 100 μl depleted extract. Material from the early purification steps corresponded to 100 equivalents of original membranes, and that from the eluted fractions of the S-Sepharose column to 5 times as much. Proteoliposomes were reconstituted by dialysis, collected by centrifugation in a microfuge and washed once. Crosslinking with the 86mer of preprolactin and CMC was as described¹⁷.

deduced from cloning of the corresponding complementary DNAs from MDCK and HeLa cells, is indicative of a multispanning membrane protein. It is at least as abundant as ER membrane-bound ribosomes. With an improved reconstitution system having an overall transport efficiency approaching that of the original microsomes, we show that TRAM is stimulatory or required for the translocation of several secretory proteins.

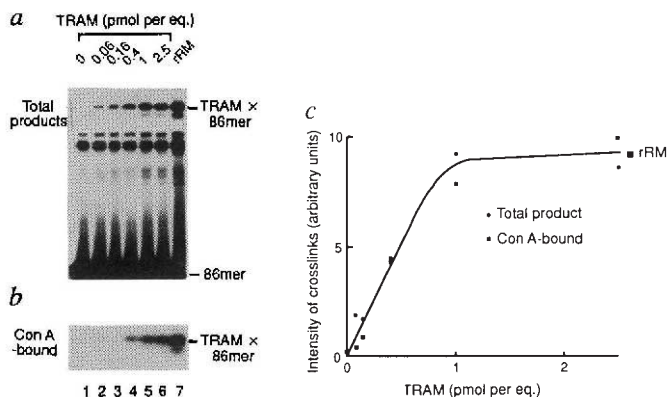
Crosslinks to SSR α

When treated with carbodiimides, nascent chains of the secretory proteins preprolactin or pre- β -lactamase translocating *in vitro* through microsomes of canine pancreas give major crosslinks

with neighbouring glycoprotein(s), as demonstrated by binding of the products to concanavalin A-Sepharose (Fig. 1*a*, lanes 3 and 10; Fig. 1*b*, lanes 3 and 8). The proportion of crosslinks to SSR α was less with short chains and more with longer ones. Thus with fragments of preprolactin containing 86 or 132 amino acids, about 2% and 10%, respectively, could be immunoprecipitated with peptide-specific antibodies against SSR α (Fig. 1*a*, lanes 5, 7, 12 and 14). Similarly, with a fragment of pre- β -lactamase of about 70 residues, only small amounts, if any, could be immunoprecipitated (Fig. 1*b*, lane 5), whereas with a fragment of 141 amino acids crosslinks to SSR α constituted a significant proportion (about 30%) (lane 10). These results sug-

FIG. 3 *a*, Crosslinked products produced by CMC with the 86mer of preprolactin and reconstituted proteoliposomes containing increasing amounts of purified TRAM. rRM, control proteoliposomes reconstituted from an unfractionated extract. *b*, Same experiment as in *a*, but products bound to con A. Note the presence of a minor crosslinked product of partially proteolysed TRAM. *c*, Quantitation of data shown in *a* and *b*.

METHODS. The amount of TRAM added was estimated from the intensity of the Coomassie-stained band in an SDS gel using glyceraldehyde phosphate dehydrogenase as standard. Reconstitution and crosslinking are described in the legends to Figs 1 and 2. Quantitation of crosslinked product was by scanning X-ray films of different exposure times.



gest that the products are heterogeneous, with most crosslinks of short chains containing a glycoprotein other than SSR α of about the same apparent size.

Purification of TRAM

To purify the predominant partner of short nascent chains, crosslinking and reconstitution methods were combined. Proteoliposomes reconstituted from a cholate extract of canine microsomes by dialysis¹⁸ gave the same crosslinked product as authentic microsomes when tested with the 86-residue fragment of preprolactin and the reagent carbodiimide (Fig. 2b, compare lanes 10 and 11). The product disappeared if the extract was first depleted of glycoproteins before dialysis (Fig. 2b, lane 1) and reappeared after replenishment with the total glycoprotein fraction (lane 2). The glycoprotein crosslinking partner was purified further by ion-exchange chromatography (Fig. 2a shows the protein pattern of the different fractions, and Fig. 2b the corresponding assays). A homogeneous protein with an apparent size of 36K was obtained (Fig. 2a, lane 10) and termed translocating chain-associating membrane protein. The signal peptidase complex¹⁹ is separated from this TRAM protein dur-

ing the last chromatography step (Fig. 2a, principal bands of low molecular weight in lane 7). As assessed by immunoblotting with different antibodies, purified TRAM still contained traces of ribophorins and signal peptidase (0.5% and 1–2%, respectively, compared with microsomes), whereas the receptor for the signal recognition particle and SSR were undetectable.

TRAM was the only protein that needed to be added to the concanavalin A-Sepharose flow-through fraction in order to achieve crosslinking (Fig. 2b, lane 9). This was confirmed by a titration experiment (Fig. 3) in which proteoliposomes with increasing amounts TRAM were tested for crosslinking with the 86-residue preprolactin fragment. Maximal yields of product were obtained with ~1 pmol TRAM per unit equivalent of microsomes, corresponding roughly to 0.5–1 pmol membrane-bound ribosomes⁵.

In most experiments there were proteolytic fragments of TRAM that had the same N-terminal amino-acid sequence and were still able to associate with short nascent chains (data not shown; see also the crosslinks of lower molecular weight in Figs 2 and 3).

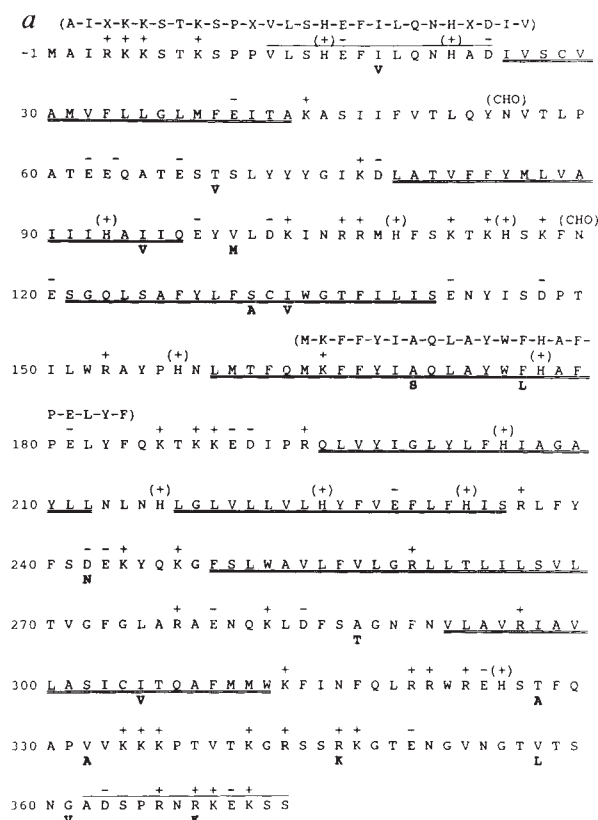


FIG. 4 **a**, Deduced amino-acid sequences (single-letter code) of TRAM in MDCK and HeLa cells obtained by cloning of cDNAs. In the sequence from HeLa cells deviating residues are shown in bold face below that from MDCK cells. Above are shown in parentheses amino-acid sequences directly determined from dog pancreatic TRAM. Lines above the sequence indicate peptides against which antibodies were raised in rabbits. Putative membrane anchors are doubly underlined. Potential carbohydrate attachment sites are indicated by (CHO). **b**, Predicted membrane topology of TRAM. **c**, Verification of the cloned cDNA. Lanes 1–4, Photocrosslinking with the 86mer of preprolactin in the absence or presence of microsomes. The products were either subjected directly to SDS-PAGE or immunoprecipitated with affinity-purified antibodies raised against a synthetic peptide corresponding to the C-terminus of TRAM (anti-TRAM). In a control, excess of antigenic peptide was added (comp. peptide). Lanes 5–8, crosslinking with CMC of the signal recognition particle-arrested fragment of pre- β -lactamase (β -lactamase AF) and microsomes. Total products and the immunoprecipitates were analysed by SDS-PAGE. Lane 9, Immunoblot analysis of microsomes (RM) with affinity-purified anti-TRAM antibodies.

METHODS. Purified TRAM was either subjected directly to automated sequencing or cyanogen bromide peptides were produced, separated by SDS-PAGE, blotted onto PVDF membranes and subjected to automated sequencing¹⁷. A cDNA library from MDCK cells was screened with degenerate oligonucleotides corresponding to the peptides. Positive clones were sequenced in both strands. A polymerase chain reaction fragment of about 500 bp in the coding region was labelled by random priming and used to screen a HeLa cell cDNA library. Prediction of the membrane topology was carried out with the PCGene computer programs. Antibodies were raised in rabbits with peptides coupled to keyhole-limpet haemocyanin. Antibodies were affinity-purified on a column containing coupled peptide¹⁷. For photocrosslinking, the 86mer of preprolactin was synthesized in the wheat-germ translation system in the presence of 4-(3-trifluoromethyldiazirino)benzoic acid-labelled lysyl transfer RNA and crosslinked as described^{11,17}. For immunoprecipitation from 1 μ l translation mixture, 10 μ g Sepharose-coupled anti-TRAM immunoglobulin was used. Immunoprecipitation after crosslinking with CMC was with 15 μ l translation mixture and 30 μ g matrix-coupled immunoglobulin. The cDNA sequences are available from the EMBL data base under the accession numbers X63678 and X63679.

Sequence and topology

Based on the sequences of the N terminus and of an internal peptide of TRAM (Fig. 4a), oligonucleotide probes were synthesized and used to screen for cDNA clones in an MDCK cell (derived from canine kidney) library. Positive clones were found that contained the entire coding region of 376 amino acids, including that for the expected peptides (Fig. 4a). A highly homologous sequence was found in a human HeLa cell (derived from a cervical carcinoma) cDNA library. The sequence had no obvious similarity with any other protein in the database.

The amino-acid sequence indicates that TRAM probably crosses the membrane eight times (Fig. 4b). Some of the membrane anchors contain a number of hydrophilic and charged residues (Fig. 4a). Some 60 amino acids at the C terminus must be located in the cytosol, as judged from proteolysis (data not shown). The carbohydrate chain is probably attached to Asn 55 (Asn 119 would be very close to the membrane).

To confirm the identity of the cloned sequence with that of the crosslinking partner, we used antibodies for immunoprecipitation that had been raised against a synthetic peptide corresponding to the 12 C-terminal amino acids deduced from the cDNA sequence. The affinity-purified antibodies reacted only with TRAM (Fig. 4c, lane 9). For crosslinking we used the 86-amino-acid fragment of preprolactin into which photoreactive lysine derivatives had been incorporated during its synthesis¹⁰ (lanes 1–4). The yield of crosslinked product containing the 54K polypeptide of the signal recognition peptide (SRP54 × 86mer) was greatly diminished if membranes were added (compare lanes 1 and 2). A crosslinked product containing TRAM appeared at the same time (TRAM × 86mer, lane 2) that could be immunoprecipitated almost quantitatively (lane 4), demonstrating that the nascent chain had been transferred from the signal recognition particle to TRAM. Note that TRAM is the principal crosslinking partner of the membrane-inserted fragment. The antibodies also precipitated the crosslinked product

produced with a short fragment (AF) of pre- β -lactamase and the carbodiimide reagent (Fig. 4c, lanes 6 and 8). Both crosslinking techniques indicate that TRAM is close to different short nascent chains.

TRAM is abundant

Antibodies directed against a peptide sequence close to the N terminus (Fig. 4a) were used for quantitative immunoblotting¹⁵ (data not shown). It was estimated that one equivalent of canine microsomes contains ~1 pmol TRAM. These estimates fit well with the results of the titration experiment shown in Fig. 3.

TRAM is required for translocation

To investigate the components involved in translocation, we used an improved reconstitution system to increase the amount of protease-protected translocated products¹⁸; our proteoliposomes now had an overall translocation efficiency approaching that of authentic microsomes. When tested with preprolactin, the prolactin produced was protected against protease by more than 50% (Fig. 5, lanes 2 and 5). These vesicles were capable of core glycosylation of prepro- α -factor (Fig. 5, lanes 8, 11; lane 14 shows binding of products to concanavalin A-Sepharose), although they were not quite as efficient as microsomes (Fig. 5, lanes 9, 12, 15); the previous system did not have this property²⁰. A sizeable portion of signal-cleaved pro- α -factor was accessible to protease (lanes 8 and 11), in contrast to the glycosylated forms or prolactin; this form of pro- α -factor may have been produced at the cytoplasmic face of the membrane by falsely oriented signal peptidase or by back-translocation of precursor cleaved in the lumen.

To determine the importance of TRAM in translocation in this system, we depleted a detergent extract of canine microsomes of glycoproteins on a concanavalin A-Sepharose column. Samples were then replenished with the purified protein or with a partially purified signal peptidase fraction (Fig. 2a, lane 7) or with both, and proteoliposomes were reconstituted by removal of the detergent. The vesicles contained only a subset of proteins present in microsomes (Fig. 6a, lane 7) and differed slightly from control vesicles (lane 2), but did not differ significantly from each other (lanes 3–6), ruling out the trivial possibility that the added protein fractions influenced the assembly of the proteoliposomes. Proteoliposomes depleted of glycoproteins or replenished with only signal peptidase did not give crosslinked products when tested with the 86-amino-acid fragment of preprolactin in photocrosslinking experiments, indicating that most TRAM had been removed (Fig. 6b, lanes 3 and 5). In contrast, control samples or vesicles replenished with TRAM gave strong crosslinks (lanes 2, 4, 6 and 7).

For translocation tests these proteoliposomes were used in subsaturating amounts. Vesicles depleted of glycoproteins showed little or no activity for prepro- α -factor or pre- β -lactamase, as determined by the amount of protease-protected material (Fig. 6c, lanes 10). The same vesicles had a significant activity for preprolactin. Addition of TRAM alone or with signal peptidase greatly stimulated translocation of prepro- α -factor (10–100-fold in different experiments) and pre- β -lactamase (4–15-fold), and moderately stimulated translocation of preprolactin (2–5-fold) (lanes 11 and 13). The translocation efficiency of vesicles replenished with TRAM often approached that of control proteoliposomes (lane 9, and see Fig. 6d), but not consistently so (see experiment with prepro- α -factor shown in Fig. 6c). Also, the extent of protease protection was about the same (compare pre- β -lactamase, lanes 2 and 9, 6 and 13). The increase in the amount of translocated product with pro- α -factor and β -lactamase was dependent on the amount of TRAM in the proteoliposomes (Fig. 6d). Again, about 1 pmol TRAM per equivalent was needed for maximal translocation. TRAM did not restore glycosylation of prepro- α -factor (Fig. 6c, lanes 11 and 13). Signal peptidase did not stimulate translocation (Fig. 6c, lanes 10 and 12) but cleavage of the signal peptide (lanes

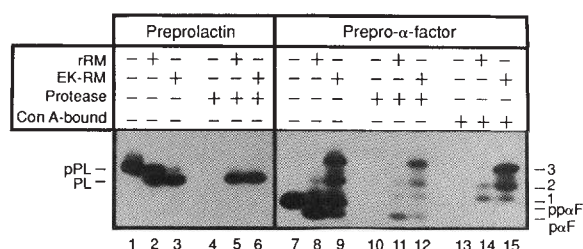


FIG. 5 Translocation of preprolactin and prepro- α -factor into proteoliposomes reconstituted from a BigCHAP-detergent extract of canine microsomes. Products were tested for protection against protease or for glycosylation (by binding to con A) as indicated. rRM, reconstituted proteoliposomes; EK-RM, control microsomes washed with EDTA and high salt; pPL, PL, preprolactin and prolactin, respectively; pp α F, p α F, prepro- α -factor and pro- α -factor, respectively; the number of carbohydrate chains attached to p α F is indicated.

METHODS. EK-RM were solubilized at 1.5 equivalents per μ l in 50 mM HEPES/KOH, pH 7.6, 400 mM potassium acetate, 2 mM DTT, 2 mM MgCl₂, 20% (w/v) glycerol, 3–4.5% BigCHAP (Calbiochem). The extract was centrifuged for 40 min in a TL 100.3 rotor (Beckman) at 95,000 r.p.m. For reconstitution, 100 μ l extract were incubated overnight with 100 mg pre-washed SM2 beads (BioRad). The vesicles were diluted 1:10 with 50 mM HEPES/KOH, pH 7.6 and sedimented in a TL 100.3 rotor (Beckman) for 40 min at 50,000 r.p.m. They were washed once and resuspended in 25 μ l. For the translocation tests, 1 μ l was added to 10 μ l reticulocyte lysate translation mixture (Promega). Transcripts coding for full-length preprolactin or prepro- α -factor were produced *in vitro* from linearized plasmids. Post-translational proteolysis was with 0.5 mg ml⁻¹ proteinase K for 30 min on ice. After addition of 5 mM phenylmethanesulphonyl fluoride, proteins were precipitated with 60% ammonium sulphate. Con A-binding was as described¹⁷, using twice as much material as for the other tests.

11 and 13), as expected. Using proteoliposomes containing purified TRAM, signal peptidase and fractionated non-glycoproteins, we found that among the latter at least the receptor for the signal recognition particle is necessary for crosslinking to TRAM and for translocation (data not shown; see also ref. 21).

The removal of glycoproteins was incomplete, as indicated by residual signal peptide cleavage (for example, see prepro- α -factor in Fig. 6c, lane 3) and variable residual translocations. In some experiments enough oligosaccharyltransferase containing the two ribophorins as glycosylated subunits (D. Kelleher, G. Kreibich and R. Gilmore, personal communication) must have been left to restore glycosylation of prepro- α -factor after replenishment with purified TRAM (Fig. 6e, compare lanes 3 and 4). Proteoliposomes immunodepleted of TRAM (about 80% could be removed) had their capacity to glycosylate reduced, but this could be reversed by replenishing with TRAM (data not shown). As glycosylation is another feature of faithful translocation, these data are further evidence that TRAM is involved in it. How TRAM influences glycosylation is still to be investigated.

Discussion

We have identified and purified a membrane protein of the mammalian ER membrane, named TRAM, which is stimulatory or required for the actual process of membrane transfer of secretory proteins. TRAM is in direct contact with translocating nascent chains after their release from the signal recognition particle and insertion into the membrane and so is involved early in translocation. But as TRAM is present in ER membranes in amounts at least equivalent to membrane-bound ribosomes, it seems to be a constituent of each translocation site and may be needed throughout the entire process of polypeptide transfer. TRAM is in contact with the translocating nascent chains and stimulates their transfer. The only other components so far for which both these requirements have been met are MOM38 (GIP,

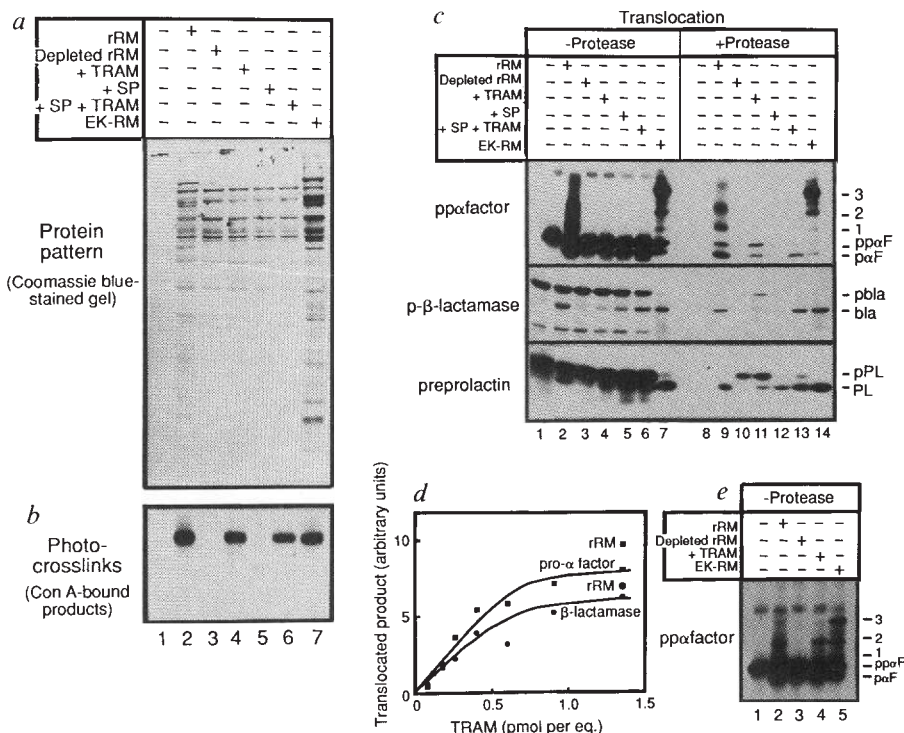
ISP42), which is involved in mitochondrial protein import²²⁻²⁵, and Sec61p and 62p, involved in secretory protein translocation in *S. cerevisiae*^{8,9,26,31}.

TRAM is a prominent neighbour of short translocating chains of different secretory proteins, as demonstrated by photocrosslinking with the probes incorporated into the nascent chain and by crosslinking with chemical reagents, whereas crosslinks to SSR α constitute only a small proportion. The similar properties of the two membrane proteins TRAM and SSR α , such as their apparent molecular mass, the sizes of the cytosolic tails, their similar abundance and the fact that they are both glycosylated, explains why it was previously believed that only one protein was responsible for the crosslinks.

Although TRAM stimulates the translocation of all secretory proteins tested, the effects varied, being only moderate for preprolactin. The situation may be analogous to that in yeast, in which different translocation substrates seem to have different requirements for components of the cellular targeting (the signal recognition particle and its receptor)²⁷ and translocation apparatus (Sec-proteins)^{28,29}. Perhaps different pathways are used. Translocation sites lacking TRAM may have a low activity for preprolactin and be inactive for other proteins. Alternatively, the translocation substrates may have different affinities for a common translocation site. Depletion of TRAM may lower the concentration of translocation sites to the extent that only proteins with a high affinity for them, like preprolactin, can still be translocated. Even if the quantities of TRAM are too low to see crosslinks in an assay requiring a stoichiometric association with the nascent chain (Fig. 6b), translocation may have still occurred if one molecule of TRAM supported the transport of several preprolactin molecules. Some TRAM was probably still present because other glycoproteins, such as signal peptidase, were incompletely removed. In agreement with this, glycoproteins are required for translocation of preprolactin into proteoliposomes reconstituted from a cholate extract (cholate, in contrast

FIG. 6 TRAM is required for translocation. *a*, Protein patterns of unfractionated reconstituted proteoliposomes (rRM), of proteoliposomes depleted of glycoproteins (depleted rRM) or of depleted vesicles replenished with TRAM (+TRAM), with signal peptidase (+SP) or with both (+TRAM + SP). EK-RM, microsomes washed with EDTA and high salt. *b*, Photocrosslinking with reconstituted proteoliposomes and the 86mer of preprolactin; the con A-bound products are shown. *c*, Cotranslational translocation of secretory proteins into reconstituted proteoliposomes. After translation, samples were incubated with or without protease and subjected to SDS-PAGE and autoradiography. pbl α and bla, Pre- β -lactamase and β -lactamase, respectively; other labels as in Fig. 5. *d*, Translocation of prepro- α -factor and pre- β -lactamase into proteoliposomes containing SP and increasing amounts of TRAM. Protease-protected material was quantitated. For comparison, the level of translocation into rRM is shown. *e*, An extract partially depleted of glycoproteins was used for translocation of prepro- α -factor as in *c* to demonstrate restoration of glycosylation by TRAM (lane 4 versus lane 3).

METHODS. BigCHAP extract (1 ml; see Fig. 5) was depleted of glycoproteins by incubation with 200 μ l con A-Sepharose overnight at 4 °C followed by a second incubation with 200 μ l resin for 4 h at room temperature. TRAM-, or SP-containing fractions from the S-Sepharose purification step were diluted with 2 volumes 50 mM HEPES/KOH, pH 7.6, 10% glycerol, and rechromatographed on a 0.5-ml S-Sepharose column. Elution was with a linear salt gradient in 50 mM HEPES/KOH, pH 7.6, 10% glycerol, 0.5% BigCHAP. The concentration of TRAM was determined spectroscopically on the basis of its known amino-acid composition (1.4 mg ml⁻¹), that of SP from its absorption (0.7 A₂₈₀ units $\approx$ 0.7 mg ml⁻¹). For 100 μ l



depleted extract, 2.5–5 μ l TRAM and 2–5 μ l SP were used. All other procedures were as described for Fig. 5, except that the proteoliposomes were used at about one-third of the concentration in translocation tests. Transcripts coding from pre- β -lactamase were obtained from Promega. Photocrosslinking is described in Fig. 4 legend.

to our detergent (Big CHAP), does not inhibit binding to concanavalin A-Sepharose¹⁸.

Although translocation into depleted proteoliposomes could be restored by replenishment with TRAM alone, it is premature to exclude the participation of other residual glycoproteins, such as the SSR complex, the signal peptidase complex or the oligosaccharyltransferase, but at least the enzymatic activities of the two enzymes are not required.

The primary structure of TRAM shows that the putative membrane-imbedded portions of the protein contain a number of hydrophilic and charged amino-acid residues. These may form or contribute to a hydrophilic environment through or along which polypeptides can be transported. Nascent translocating chains crosslinked to the membrane-imbedded domain

of TRAM but not to the cytoplasmic C terminus, for fragments shortened at the C terminus still gave crosslinks, and proteolysis after crosslinking shortened the product to an extent corresponding to the cytosolic tail¹¹⁻¹³. Whether TRAM forms a channel by itself is unclear. We consider it more likely that it is a component of a translocation complex that contains several proteins, perhaps including homologues of the yeast Sec proteins.

We have developed a much improved reconstitution system which reflects the behaviour of intact microsomes and fulfills all three criteria for translocation: signal peptide cleavage, a high degree of protease protection, and glycosylation. This system paves the way for reconstituting the mammalian translocation machinery from purified components. □

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LETTERS TO NATURE

Encapsulation of a scandium trimer in C₈₂

Hisanori Shinohara*, Hiroyasu Sato*, Masato Ohkohchi†, Yoshinori Ando‡, Takeshi Kodama‡, Tadamasa Shida‡, Tatsuhisa Kato‡ & Yahachi Saito§

* Department of Chemistry for Materials, Mi'e University, Tsu 514, Japan

† Department of Physics, Meijo University, Nagoya 468, Japan

‡ Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan

§ Department of Electrical and Electronic Engineering, Mi'e University, Tsu 514, Japan

FULLERENES with metals encapsulated within the carbon cage have been prepared recently¹⁻⁶. Laser vaporization of a lanthanum-impregnated graphite rod yielded an air-stable, solvent-extractable species comprising a single La atom inside a C₈₂ cage¹, denoted La@C₈₂; a cluster containing two La atoms (La₂@C₈₂) was later reported³. Similar techniques applied to graphite/yttrium rods have produced Y@C₈₂ and Y₂@C₈₂ (refs 5, 6). Here we describe the preparation of scandium-containing C₈₂ species, including an encapsulated scandium trimer, Sc₃@C₈₂. Mass spectrometry and ESR spectroscopy provide evidence that the Sc₃ trimer is indeed trapped within the cage.

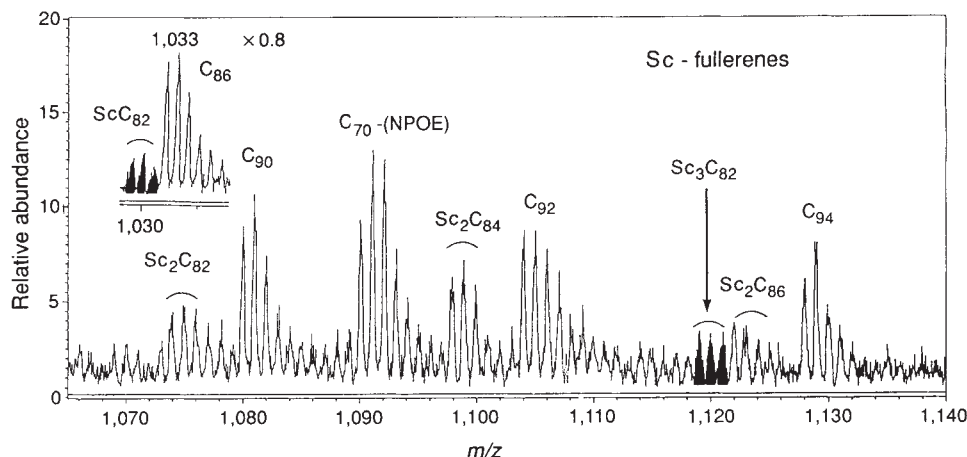
We prepared scandium-containing fullerenes by arc burning of a composite rod composed of Sc₂O₃ (99.9% purity; 2.1 g), graphite powder (99.995%; 3.6 g) and high-strength pitch (5.0 g). The scandium-graphite rods were cured and carbonized at 1,000 °C for 5 hours in vacuum (10⁻³ torr) and then baked at 1,200 °C for a further 5 hours (10⁻⁵ torr). The composite rods were used as positive electrodes which were consumed preferentially in the direct current spark mode under a static helium atmosphere (50 torr). The resulting soot was extracted in toluene,

pyridine and carbon disulphide. The extracts were analysed by fast atom bombardment and laser-desorption mass spectrometry (FAB-MS and LDMS) and ESR spectroscopy^{2,5,6}.

The FAB mass spectrum for the carbon disulphide extract shows the presence of peaks due to Sc₃@C₈₂, Sc₂@C₈₄ and Sc₂@C₈₂ in addition to C₉₀, C₉₂ and C₉₄ (relevant part of spectrum shown in Fig. 1). A weaker series of peaks due to the monoscandium fullerenes, ScC_{2n}, was also observed, including Sc@C₈₂, Sc@C₈₄ and Sc@C₈₆. The most abundant scandium-fullerene in the ScC_{2n} series was found to be Sc@C₈₂ (Fig. 1 insert). The spectrum was recorded with unit mass resolution so that each fullerene clearly exhibits a group of peaks owing to ¹³C isotopes, confirming the identification of the observed peaks. Scandium atoms can more easily be incorporated in fullerenes than lanthanum or yttrium. We estimate the total amount of scandium-fullerenes in the CS₂ extract to be less than 3% (by weight) on the basis of the present FAB-MS results. Laser-desorption time-of-flight mass spectra of the same sample exhibited metallofullerene peaks similar to those in the FAB-MS spectrum.

ESR spectroscopy of the La@C₈₂ complex indicates² that the lanthanum atom is in the +3 oxidation state; the C₈₂ cage bears a corresponding 3- charge (ref. 2). ESR results for Y@C₈₂ (refs 5, 6) reveal the same ionic structure for this species. We have used ESR to characterize the nature of the scandium-containing compounds. The ESR (9.445 GHz) spectrum of the degassed CS₂ solution at room temperature shows two prominent series of peaks (Fig. 2). The first series consists of 22 narrow (0.78 gauss, width at maximum slope) equally spaced (6.25 gauss) lines of a symmetric intensity distribution centred at g = 1.9986. The observed splitting is a manifestation of the isotropic hyperfine coupling (HFC) due to the nuclei of three equivalent scandium atoms (scandium trimer). The second series, in contrast, is composed of eight much narrower (0.45 gauss), also equally spaced (3.83 gauss) absorption lines of equal intensity centred at 1.9998. The corresponding part of the spectrum is

FIG. 1 Fast-atom-bombardment (positive-ion) mass spectrum of carbon disulphide extracts (dispersed in ortho-nitrophenyl octylether (NPOE) matrix) of scandium-containing fullerenes produced by the carbon-arc generation method of a scandium-graphite composite rod. A group of signals around mass-to-charge ratio $m/z=1,091$ is due to the C_{70} (NPOE) complex. The peaks due to ScC_{82} are shown in the insert.



expanded in Fig. 3 where the eight equally spaced lines are clearly observable. The eight lines stem unambiguously from the isotropic hyperfine coupling to the $I = 7/2$ scandium nucleus, and are consistent with the earlier $La@C_{82}$ ESR results². The observed g values are similar to those reported for C_{60}^- (refs 7–11) and C_{70}^- (ref. 12) anion radicals, and for $La@C_{82}$ (ref. 2) and $Y@C_{82}$ (refs 5, 6), which range from 1.995 to 2.001 (refs 7–12), indicating that the observed scandium-fullerenes are in doublet (radical) states. The ^{43}Sc hyperfine coupling constant (3.83 gauss) is very small as compared with the reported coupling constants (60–70 gauss) for Sc^{2+} impurities in a CaF_2 or SrF_2

lattice below 30 K (ref. 13). Unusually small HFC constants have also been reported for $La@C_{82}$ (1.25 gauss)² and $Y@C_{82}$ (0.48 gauss)^{5,6}. This is indicative of the fact that the scandium atom is the +3 oxidation state (rather than +2), resulting in the ion-pair type electronic state of $Sc^{3+}C_{82}^{3-}$ as reported for $La^{3+}C_{82}^{3-}$ and $Y^{3+}C_{82}^{3-}$.

The ESR spectrum is reproduced well by a computer simulation (upper trace in Fig. 2) using the observed HFC constants and g values. The simulation was done by assuming that only two types of scandium-fullerenes, Sc_3C_{2n} and ScC_{2n} (with 3:1 abundance), are present. The mass spectral results suggest that the most probable candidates for these fullerenes are $Sc_3@C_{82}$ and $Sc@C_{82}$. Although $Sc_2@C_{82}$ and $Sc_2@C_{84}$ are also observed in the mass spectrum, there is little evidence for the presence of Sc_2 -fullerenes in the ESR spectrum. We have widely scanned the ESR spectrometer from 830 to 5,830 gauss for the present sample in the temperature range of 3–300 K and found no paramagnetic signals attributable to Sc_2 -fullerenes. The Sc_2C_{2n} type of fullerenes such as $Sc_2@C_{84}$ and $Sc_2@C_{82}$ may not be paramagnetic species. A similar negative result has been reported recently for the yttrium dimetallofullerenes, Y_2C_{2n} (ref. 6).

The symmetric hyperfine splitting of the 22 lines for $Sc_3@C_{82}$ does indicate that the three scandium ions must be equivalent within the time scale of the ESR measurements. This could reflect either fast dynamic averaging between distinct scandium-ion environments, or geometrical equivalence of the three ions (corresponding to a triangular arrangement). In either case, all three Sc ions must be trapped within the C_{82} cage. For each of

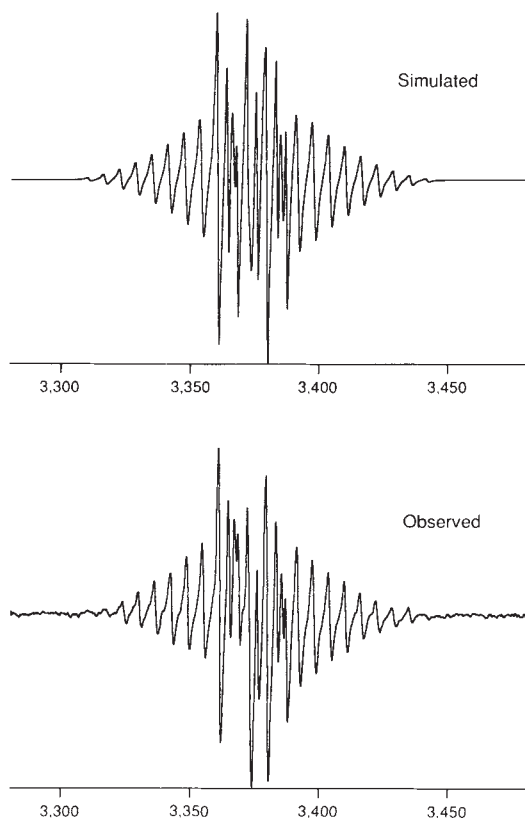


FIG. 2 ESR spectrum of the room-temperature CS_2 extract of scandium-containing fullerenes at a microwave frequency of 9.445 GHz (lower trace); the simulation spectrum (upper trace). The spectrum contains signals from two types of species (for details, see text). The prominent hyperfine splitting into 22 peaks centred at 3,376 gauss is due to Sc_3C_{82} . The sample was degassed several times by a freeze-thaw cycle and sealed in a thin-wall pyrex tube.

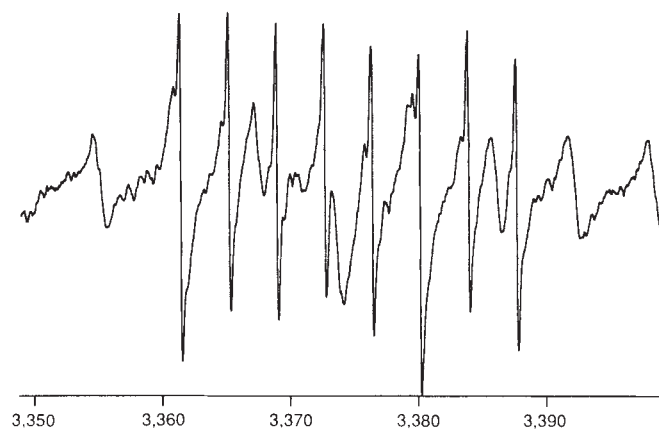


FIG. 3 ESR spectrum at room temperature of the same sample as Fig. 2, but with an expanded scale in magnetic field from 3,350 to 3,400 gauss. The hyperfine splitting into eight equally spaced peaks of equivalent intensity can be seen. Small peaks adjacent to the main lines are due to hyperfine coupling to the ^{13}C isotopes.

the three C_{82} isomers recently identified by ^{13}C NMR spectroscopy¹⁴, the empty cages have internal diameters of at least 5 Å, which provides adequate room for encapsulation of three Sc ions (a triangular Sc_3 ion has a radius of ~ 1.4 Å; up to four Sc

atoms can be incorporated into one of the isomers in terms of space). The ability to incorporate various kinds of metal cluster within fullerenes of different sizes may in future lead to materials with novel solid-state properties. □

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Domain boundaries and buckling superstructures in Langmuir–Blodgett films

J. Garnaes, D. K. Schwartz, R. Viswanathan & J. A. N. Zasadzinski*

Department of Chemical and Nuclear Engineering, University of California, Santa Barbara, California 93106, USA

* To whom correspondence should be addressed

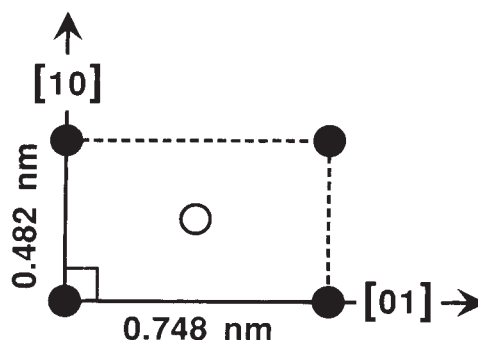
MANY technological and scientific applications have been proposed for Langmuir–Blodgett (LB) films¹. These ordered arrays of oriented amphiphilic molecules may be useful as nonlinear optical systems², as insulating or patterning layers in microelectronics^{3,4}, as model systems for studies of two-dimensional phases⁵ and as molecular templates for protein crystallization⁶. The potential of LB films for these applications is sensitive to the details of their molecular packing; in particular, they require that the layers have a defect-free, periodic structure¹. Here we present images from atomic force microscopy of domain boundaries between regions of different crystallographic orientation in LB multilayers. The regular lattice structure is preserved to within 1 nm of the grain boundaries, and the domains are oriented in a near-twinning arrangement. We also observe a periodic buckling

superstructure along a particular lattice symmetry direction, with a wavelength of about 2 nm and an amplitude of ≤ 0.1 nm. The buckling was independent of surface pressure during deposition, dipping direction, number of layers deposited and nature of the substrate, and was stable over many hours. These departures from two-dimensional periodicity may have an important bearing on applications that rely on perfect crystallinity.

LB and other organic thin films suffer from molecular-scale defects such as pinholes, dislocations and grain boundaries which are the limiting factor in many applications^{1–4}. For example, conduction through LB monolayers and multilayers used as insulators is believed to be dominated by defects, especially grain boundaries³. The quadratic enhancement of second-harmonic generation possible in LB multilayers depends on oriented and layered arrays of charge-transfer complexes, often interlayered with spacer molecules; packing defects can disrupt the dipole ordering and eliminate the quadratic enhancement⁷. Fundamental studies of molecular ordering in LB films may provide unique insights into the thermodynamics of two-dimensional systems with multiple degrees of freedom^{5,7,8}. LB films are also excellent models with which to study the organization and interactions of lipids and proteins in biomembranes⁴, as well as molecular templates for protein crystallization⁶. Most experimental techniques used to study LB films are not, however, sensitive to defect structures at molecular resolution^{9–20}.

The bulk structure and symmetry of LB films have been studied extensively by electron diffraction^{9–13}, X-ray diffrac-

FIG. 1 Lattice parameters of two- to five-layer films of cadmium arachidate measured by AFM²⁷. The position of the molecule (○) is shifted slightly from the centre of the rectangle. This shift is small compared to the lattice parameters. The standard deviation of a single measurement is ~ 0.005 nm for the [10] direction and ~ 0.008 nm for the [01] direction. Possible systematic error due to the calibration (estimated to be ~ 0.003 nm) is not included. To prepare the LB film we spread arachidic acid ($\text{CH}_3(\text{CH}_2)_{18}\text{COOH}$) (Aldrich Chemical, >99% purity) from chloroform solution (2 mg ml^{-1}) on a water subphase of a commercial NIMA trough (NIMA Technology Ltd., Coventry, England) with $5 \times 10^{-4} \text{ M CdCl}_2$ and an adjusted pH of 6.5. By vertical dipping (1.6 mm min^{-1}) films were transferred to polished silicon wafers with orientation (100) (Semiconductor Processing, Boston, Massachusetts) at surface pressures of 10.0 and $30.0 \pm 0.1 \text{ mN m}^{-1}$. For films with an odd number of layers the wafer was cleaned in a hot solution of $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$. For films with an even number of layers the cleaning was followed by a 5-s etch in 10% HF, to make the substrate hydrophobic. One film was prepared on freshly cleaved mica. Details are given elsewhere²⁷. We used a Nanoscope II FM (Digital Instruments, Goleta, California) together with microfabricated Si_3N_4 tips formed on cantilevers with a spring constant of 0.12 N m^{-1} . The images (400×400 pixels) were recorded in air at room temperature using 'force mode'; that is, measuring the z-deflection of the cantilever while scanning the sample at roughly constant height. The repulsive mean force ($\sim 10 \text{ nN}$) was found not to be critical. The effect of drift was minimized by using a large scan area (up to $35 \times 35 \text{ nm}^2$) and a fast scan rate of 39 Hz. The images were stable for hundreds of scans (a period of hours) while we waited for the drift to disappear. The AFM scanner ($1,000 \times 1,000 \text{ nm}^2$) was carefully calibrated by fitting linear calibration constants in the x and y directions to images of mica²⁷.



tion¹⁴⁻¹⁹, and X-ray absorption²⁰. These techniques give structural information averaged over macroscopic length scales (much greater than a micrometre) and cannot selectively give information about a single layer of a multilayer film, or about the defect structure within a layer. Scanning tunnelling microscopy has also been applied to structural studies of organic molecules either adsorbed or deposited on substrates, but the technique is limited to conducting substrates and layers less than a few nanometres thick, so it is of limited use for studies of LB monolayers^{21,22} and cannot be used for multilayers. The atomic force microscope (AFM)²³⁻²⁷, recently used to study the surface structure of LB films, has the advantage of probing only the surface topography, and the capability to resolve individual molecules as well as defects such as dislocations and domain boundaries in the surface layer. With a limit in height detection of less than 0.01 nm, the AFM is also ideal for investigations of submolecular structures. The superstructures and domain boundaries presented here would be difficult, if not impossible, to detect by any other technique.

In the top layer of two- to five-layer films of cadmium arachidate on silicon, we found a rectangular lattice with a two-molecule basis²⁷. The lattice parameters are in good agreement with diffraction data from close-packed, untilted, aliphatic systems²⁸ and the packing density on the water subphase during deposition. The lattice structure is visible in the figures. The observed thickness of the film (obtained by measuring the depth of holes with the AFM²⁴) confirms that our images show the terminal methyl groups of the outermost layer with a height corrugation of ≤ 0.2 nm. The lattice parameters are summarized in Fig. 1.

The boundary between two distinct ordered domains with areas $>10^4$ nm² is shown in Fig. 2a. The two domains are misoriented by $55.9 \pm 0.5^\circ$ and the lattice order is maintained to within ~ 0.3 nm on each side of the boundary. The observed lattice misorientation leads to a mismatch at the boundary (Fig.

2c). A calculation shows that ~ 36 rows of molecules along the $[11]$ lattice direction will meet ~ 34 rows along the $[\bar{1}\bar{1}]$ direction. This is in agreement with our observation, and a detail of this mismatch is shown in Fig. 2b. As the molecular ordering near the grain boundary is similar to that within the grain, it is unlikely that grain boundaries are pathways of enhanced diffusion or conduction as earlier postulated³.

Two other observations of well defined adjacent domains (not shown) gave relative orientations of $58.4 \pm 0.5^\circ$ and $66.9 \pm 0.5^\circ$. Transmission electron diffraction (TED) of a three-layer film deposited on carbon-coated Formvar and cooled to 74 K showed 'hexagonal twinning': all the domains had orientations close to either 0° or $\pm 60^\circ$ relative to a fixed direction. Because the electrons scatter from all layers simultaneously, TED cannot distinguish between twinning in a single layer (for example, the top layer) or in domains in different layers. Hexagonal twinning has often been reported in substances similar to cadmium arachidate^{9,10}. These TED observations, therefore, suggest that the three relative orientations observed by AFM in the top layer were clustered around 60° not by coincidence, but because the top layer consists of domains that have relative orientations close to either 0° or $\pm 60^\circ$. By comparing the number of boundaries observed with the number of lattice images examined overall, we can estimate the domain size (based on statistical considerations) at $2 \mu\text{m}$.

We have also observed a new and unexpected feature: a distinct surface buckling with a peak-to-valley height of ≤ 0.1 nm (Fig. 3a). The two-dimensional Fourier transform in Fig. 3b shows that the buckling is within $\pm 2^\circ$ of the $[10]$ direction and has a period of 1.9 ± 0.3 nm. The two-dimensional Fourier transform in Fig. 3c shows that 'satellite' spots are also present at a wavevector corresponding to the sum of the wavevectors for the buckling and molecular lattice. This implies that the 'in-plane' position of the molecules is also modulated by the buckling.

The amplitude of the buckling wave varied from domain to

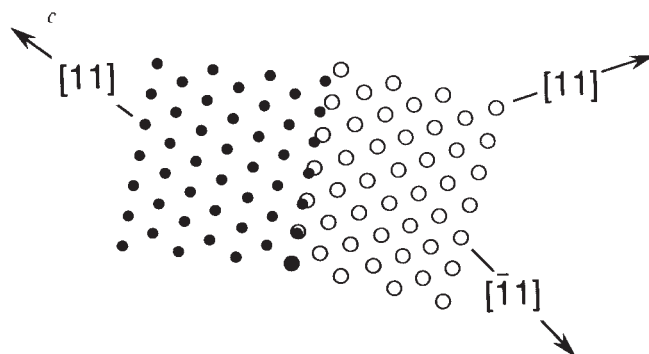
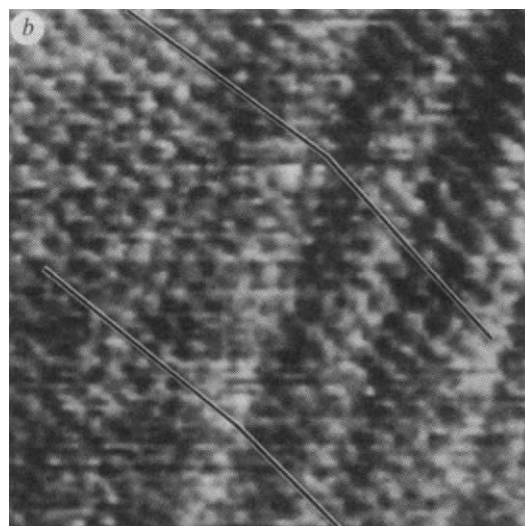
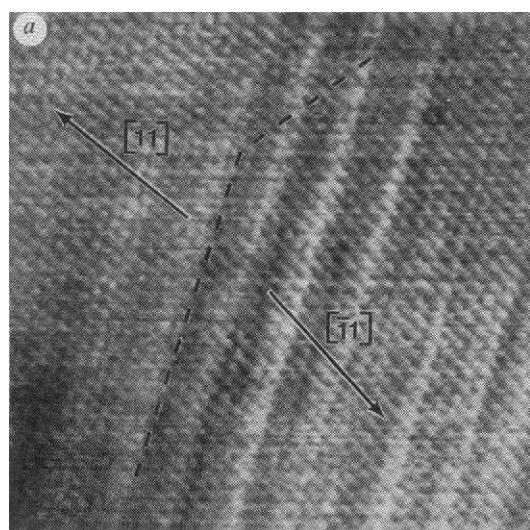


FIG. 2 a, AFM image (17.5×17.5 nm²) of a four-layer film of cadmium arachidate. The dashed line marks the boundary between two regions with different lattice orientations. Arrows indicate lattice directions in the two domains. The bright areas of the image are ~ 0.2 nm higher than the dark areas of the image. b, Magnified region (8.5×8.5 nm²) of the boundary. On the left, ten rows of molecules are between the two lines; on the right there are only nine. The boundary can be identified easily by looking at a grazing angle along the lines. Both a and b are parts of a total scan area of 35×35 nm². c, Schematic lattice structure of the observed boundary. Dots and circles represent molecules in the two observed domains.

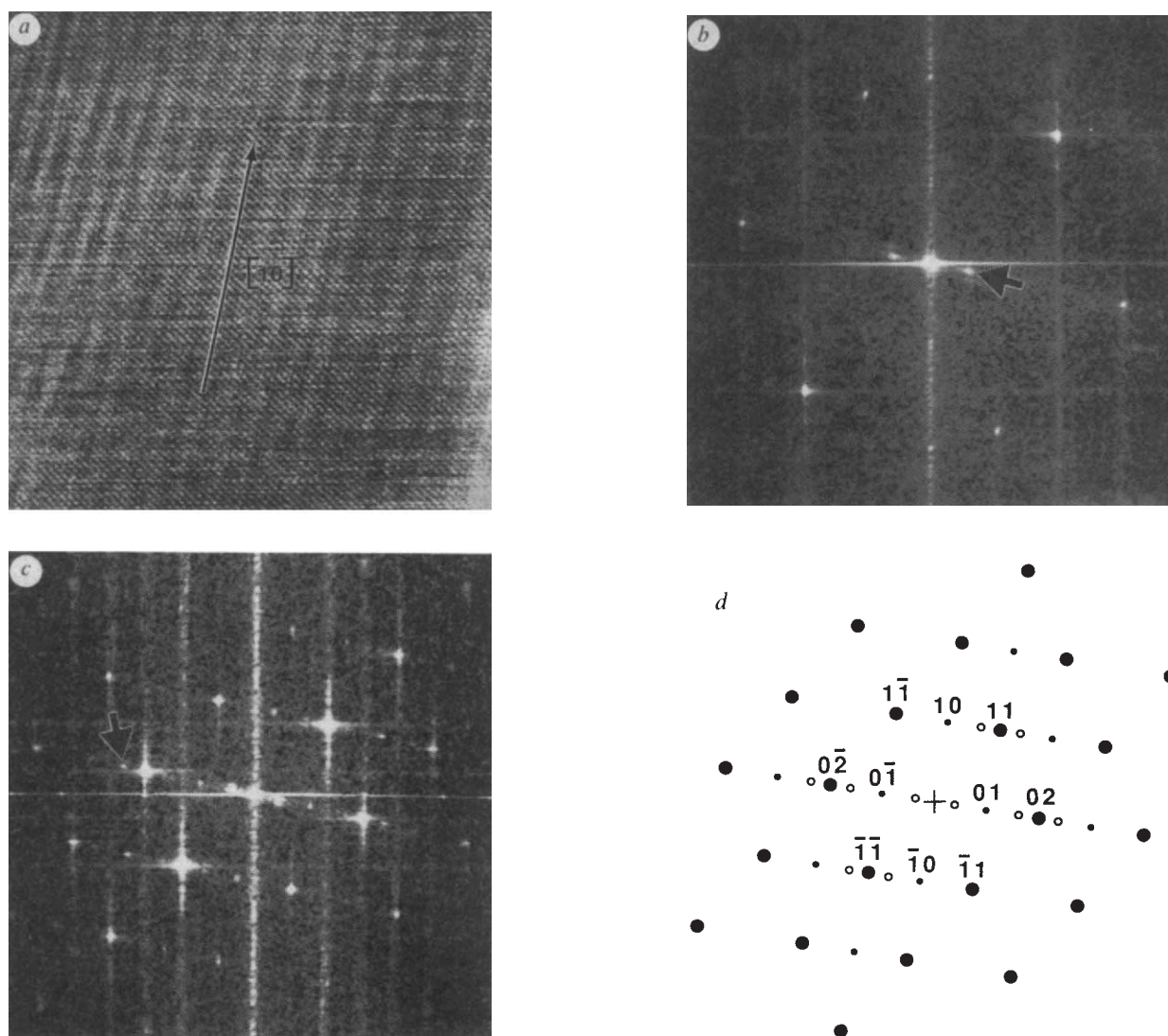


FIG. 3 AFM image ($40 \times 40 \text{ nm}^2$) of a four-layer film of cadmium arachidate showing buckling along the $[10]$ direction (the light/dark ridges parallel to the arrow). The buckling structure extended over more than $100 \times 100 \text{ nm}^2$. *b*, Two-dimensional Fourier transform of *a*. The pair of spots marked by arrows correspond to the buckling; the other six spots are the strongest spots due to the molecular lattice. The horizontal and vertical lines through the centre and the strong spots are noise due to the rastering. *c*, Two-dimensional Fourier transform of a smaller scan area of $20 \times 20 \text{ nm}^2$ (not shown here) from the same domain as *a*. The 'satellite' spots marked by the arrows correspond to the modulation of the molecular lattice distance by the buckling. *d*, Schematic diagram of all the spots in the two-dimensional Fourier transform shown in *c*. The open circles near the centre correspond to the buckling; the other open circles are the 'satellite' spots discussed above. The small filled circles correspond to the weak spots of the reciprocal

lattice (hk) where $h+k$ is odd. These spots would be missing entirely if the lattice structure were centred rectangular (see Fig. 1). The large filled circles correspond to the strong spots of the reciprocal lattice (hk), where $h+k$ is even. It can be shown, using symmetry considerations for a tip interacting with an infinite single-crystal lattice, that no finite tip structure can cause a z -deflection with a period longer than the longest repeat distance in the sample lattice (0.72 nm). An artificial z -deflection with a period larger than 0.72 nm (and extending over more than $100 \times 100 \text{ nm}^2$) can arise only from the unlikely hypothesis that the tip has 'mini-tips' separated by more than 100 nm , and that two mini-tips simultaneously trace domains with different orientations. Random bumps on the film surface with heights of $\sim 0.5 \text{ nm}$ and a typical diameter of 20 to 50 nm (due to the polished substrate) prevent such hypothetical mini-tips from simultaneously tracing the surface of the film over large areas.

domain. In Fig. 4*a*, the domain to the left has a buckling identical to that in Fig. 3, with an amplitude decreasing near the domain boundary. Both visual inspection of the image and a Fourier transformation show that there is no observable buckling along the $[10]$ direction in the domain to the right.

We observed buckling structures along the $[10]$ direction in every film of two to five layers that we examined. In 30 independent repetitions of the measurement, the buckling was present in 20 of the sampled domains. Some measurements showed a 'broadband' buckling (no distinct period) with wavelengths from 1 – 4 nm , in the $[10]$ direction. In about 60% of the domains with buckling, however, a distinct period of $2.3 \pm 0.3 \text{ nm}$ was

measured. (As the amplitude of the buckling is near the resolution limit of the AFM, it is not surprising that we do not see buckling in every image. We do, however, see the buckling in every type of multilayer system that we have examined.) The observations were done with several different tips, scan areas from $10 \times 10 \text{ nm}^2$ to $50 \times 50 \text{ nm}^2$, different sample orientations and even the use of a second scanner. The buckling was seen on films prepared on both amorphous silicon oxide and atomically flat mica substrates, which implies that neither periodicity nor random roughness on the substrate affects the buckling superstructure. Buckling was observed on films deposited from the water surface at both 10 mN m^{-1} and 30 mN m^{-1} ; its direc-

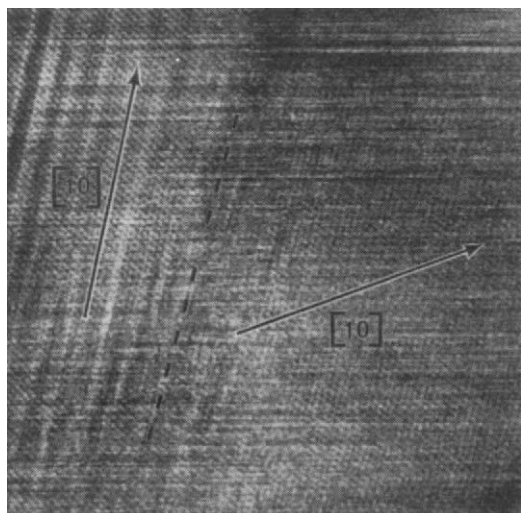


FIG. 4 AFM image ($35 \times 35 \text{ nm}^2$) of a four-layer film of cadmium arachidate, showing buckling near a domain boundary (dashed line). The domain on the left has a strong buckling along the $[10]$ direction (the light/dark ridges parallel to the arrow), whereas no buckling parallel to the arrow is seen in the domain to the right.

tion was not related to the dipping direction. The buckling was very robust, as it was observed on films that had been stored for up to three months in air as well as on films that had been allowed to sit in the water subphase for up to an hour, and was stable over hours of AFM scanning.

Although it is possible that the buckling is a non-equilibrium structure, perhaps related to stress in the film, the robustness and directionality of the structure, as well as the fact that the wavelengths cluster around 2.3 nm, suggest that it may indeed be an equilibrium superstructure. Such periodic, undulating structures ('ripple' phases) have been seen in saturated phospholipid systems²⁹ and are often related to a frustration in molecular packing due to the internal anisotropy of the molecule^{7,8}. In particular, there may be competition between the packing of the head and tail groups because of their different size or symmetry. If this were true, then the ripple would be expected to change in wavelength or disappear entirely if the surfactant were changed. Further experiments using different chain lengths, head groups of different sizes and different cations will help to clarify the issue. □

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Autocatalytic self-replicating micelles as models for prebiotic structures

Pascale Angelica Bachmann*, Pier Luigi Luisi*† & Jacques Lang‡

* Institut für Polymere, ETH-Zentrum, CH-8092 Zürich, Switzerland

† Institut Charles Sadron (CRM-EAHP), CNRS, 6 rue Boussingault, F-67000 Strasbourg, France

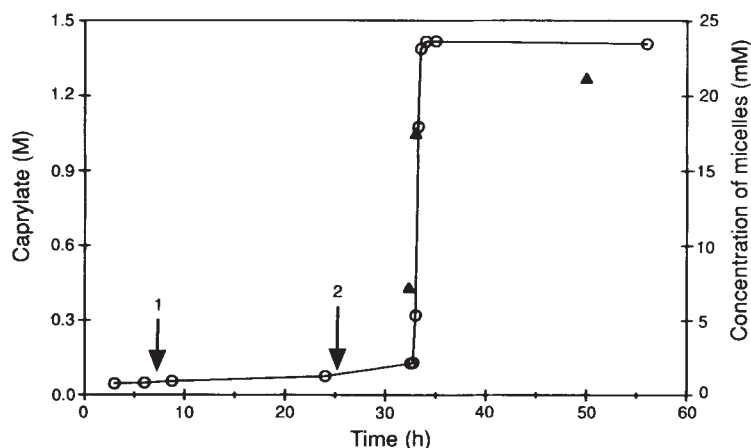
MICELLES that can catalyse their replication have been described recently^{1–3}. In the previous experiments, micelles (or bilayer vesicles⁴) were always present in the initial reaction mixture—that is, the system was presented with the bounded structures required for autocatalysis. Here we describe a system in which autocatalytic micelles are formed from amphiphiles that are themselves generated from a hydrolysis reaction in the absence of compartmental structures. Alkaline hydrolysis of ethyl caprylate (itself insoluble in water) yields sodium caprylate, initially at a very slow rate; but as soon as sufficient caprylate is formed for aggregation into micelles to take place, there is an exponential increase in reaction rate owing to micellar catalysis. These self-assembling surfactant structures may consequently provide a model system for studies of prebiotic chemistry. The possible relevance of this process to prebiotic chemistry is emphasized by our observation that the micelles can be converted into more-robust vesicles by a pH change induced by dissolved CO₂.

Unlike the self-replication of short linear molecules based on template reactions as described by von Kiedrowski⁵ and Rebek and co-workers⁶, our self-replicating micelles are structures with geometrically defined boundaries, the necessary condition for applying the term 'self-replication' being that the process leading to population growth takes place within the boundary of these parent structures. A structure that is self-bounded, and is able to self-generate due to reactions which take place within the boundary, meets the criteria of autopoiesis⁷. The term 'autopoiesis' stems from the Greek 'auto' (self) and 'poiesis' (formation). The micellar self-replicating systems can be seen in this perspective—that is, as attempts to model with simple synthetic systems the basic chemical mechanisms of life.

We use a two-phase system consisting of 20 ml of an aqueous 3M NaOH solution and a supernatant phase of 6 ml net ethyl-caprylate, which is insoluble in water. The system is stirred with a magnetic stirrer at 150 r.p.m. slightly below 100 °C under reflux, so that the two phases remain clearly separated during the reaction. Hydrolysis of the ester yields ethanol and the amphiphile sodium caprylate, the latter known to form micelles in water⁸. When the reaction reaches completion after ~34 h, a single transparent micellar phase is obtained. The development of the reaction with time is shown in Fig. 1: the formation of caprylate is sluggish until the critical micelle concentration is reached (determined to be 0.1 M under our conditions), after which the rate increases exponentially. This considerable increase is due to a micellar catalytic process. When micelles are formed, each rapidly binds to an average of five

† To whom correspondence should be addressed.

FIG. 1 Time changes in the caprylate concentration (○) and micellar concentration (▲) following the hydrolysis of ethylcaprylate (EC) by NaOH. The two-phase system consisted of 20 ml aqueous 3 M NaOH solution and a supernatant phase of 6 ml solution of net EC. The system is stirred at 150 r.p.m. slightly below 100 °C under reflux, the phase separation remaining clearly visible during reaction. Samples were withdrawn at time intervals for analysis. The caprylate concentration was determined by Fourier-transform infrared (FTIR) spectroscopy using the increasing intensity of the strong C=O band at $1,570\text{ cm}^{-1}$. The micellar concentration was measured by time-resolved fluorescence quenching with pyrene as the fluorescent probe and decylpyridinium as the quencher¹⁰. FTIR and fluorescence methodologies are described in our previous paper³. Onset of the exponential growth (the point at which the micellar catalysis begins) was regulated by increasing the stirring speed to 450 r.p.m. (first arrow, onset at 7–8 h), reflecting the dependence of the hydrolysis rate on the surface area. Addition of 20 mM sodium caprylate to the initial reaction mixture also produced a reduction in the time to onset (second arrow, onset at 25 h). When caprylate is already present, a shorter time is needed to reach the critical micelle concentration. This agrees with a linear extrapolation of the first reaction phase from which an onset time of 25.6 h is calculated.



ethylcaprylate molecules (see Fig. 2), significantly increasing the rate of hydrolysis. Thus, new micelles are produced by a chemical reaction that takes place within the domain of the parent micelles: an example of autopoietic self-replication.

It is possible to quantify the acceleration effect brought about by micellar catalysis. Each of the two reaction phases (slow and fast) corresponds to a bimolecular process, as can be shown by data linearization⁹. The bimolecular rate constant for the non-catalysed process is $2.1 \times 10^{-4}\text{ M}^{-1}\text{ s}^{-1}$, and that of the catalysed reaction $0.19\text{ M}^{-1}\text{ s}^{-1}$, an increase by a factor of ~ 900 . This enhancement is most probably due to the large increase of the surface area in the micellar solution and the milieu-effect: the reaction in the micelles takes place not in bulk water, but in a more hydrophobic environment.

The increase in micelle population can be monitored directly by time-resolved fluorescence quenching. This method is

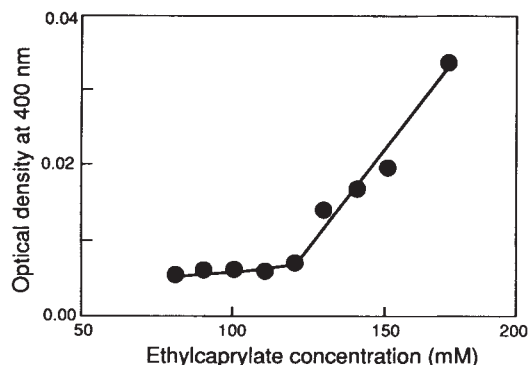


FIG. 2 Quantitative analysis of the binding of ethylcaprylate to sodium caprylate micelles in water by UV-turbidimetric titration using a 1-cm optical path length and a 400-nm probe beam. The water insoluble ethylcaprylate is added to an aqueous solution of sodium caprylate micelles. The ester is solubilized in the micelles giving rise to a transparent single-phase solution. Once the micelles are saturated by ethylcaprylate, the optical density of the solution increases, because of scattering from large aggregates of the water insoluble ester. We also quantified the uptake of ester by FTIR, detecting the intensity of the strong C=O absorption band of the ester (data not shown). Both methods consistently indicated that a maximum of 120 mM EC are bound to 1.5 M sodium caprylate in water. The average aggregation number for the caprylate micelles under our experimental conditions is 60, giving an average of 5 EC molecules (8%) per micelle.

described in ref. 10, and has been used in our previous work on self-replicating reverse and aqueous micelles¹⁻³. From Fig. 1, it can be seen that the micelle concentration increases from 0 to 21 mM during the course of the reaction.

Aqueous micelles of caprylate ions can be transformed into vesicles, although not very stable ones, by decreasing the pH to ~ 6.5 ^{11,12}. We have verified this by titrating our micellar reaction solution on completion of the hydrolysis with HCl down to pH 6.7; using freeze-fracture electron microscopy, we observe vesicles of $\sim 150\text{ nm}$ radius (see Fig. 3). A decrease in pH can also be achieved by addition of gaseous CO_2 to the micellar

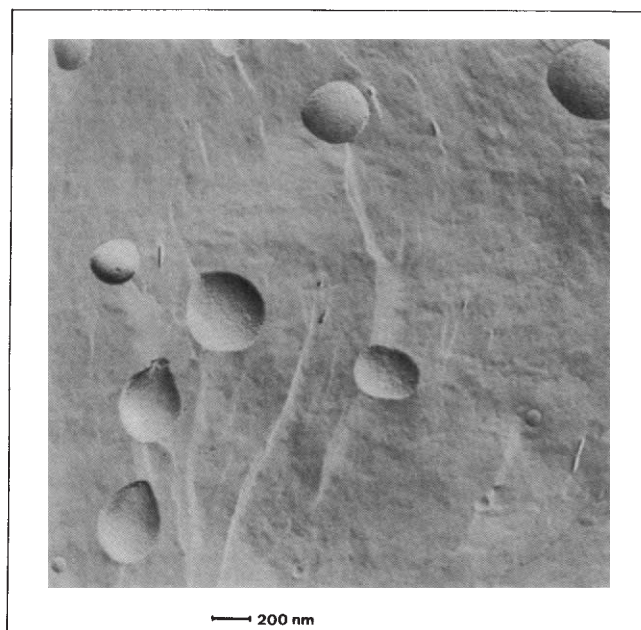
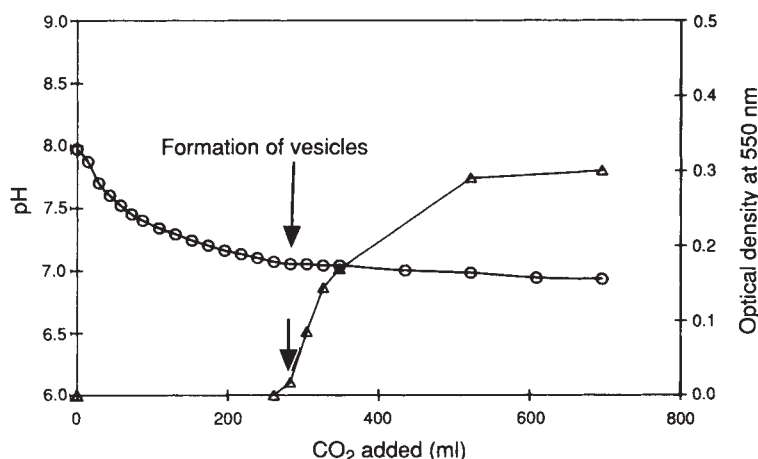


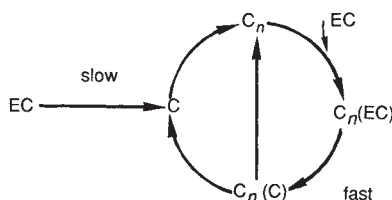
FIG. 3 Freeze-fracture electron microscopy of caprylate vesicles. Electron micrographs were taken after freezing the samples by the propane-jet technique^{14,15}. The cryofixed samples were fractured at 108 K in a Balzen BAF 300 at a pressure of 10^{-5} Pa . Platinum/carbon replicas were produced and examined in a Philips EM 301 microscope at 100 kV. Photomicrographs were taken on Agfa Scientia 23D56 cut films and developed in Geratone G5C for 3.5 min at 293 K.

FIG. 4 Decrease of pH (○) and increase of turbidity (△) arising from addition of CO₂ to a micellar caprylate solution. The micellar solution consisted of 0.34 M caprylate in 0.34 M aqueous ethanol. CO₂ was added at a flow rate of 87 ml min⁻¹ using a glass tube inserted into the solution. The solution was stirred at 250 r.p.m. and the pH was continuously measured. Samples were withdrawn at time intervals and the turbidity measured by ultraviolet spectroscopy with a 1-cm optical path length and a 550-nm probe beam. This system has two important differences to that in Fig. 1, with the concentration of EC lower by a factor of 4 and an equimolar concentration of NaOH present in the aqueous phase. Under the modified conditions, it was possible to reduce the pH down to 7 without precipitation of Na₂CO₃.



solution, simulating a change caused by atmospheric agents. The results of bubbling about 300 ml of CO₂ through 20 ml of a micellar caprylate solution are shown in Fig. 4: as soon as the pH drops to ~7, vesicles are again formed.

The overall process of Fig. 1 can be illustrated as follows (EC representing ethylcaprylate; C caprylate; C_n the micelle, supposed for simplicity to be monodisperse):



The process is characterized by micellar catalysis (the fast hydrolysis step within the species C_n(EC)), whereas the whole cycle can be seen as autocatalytic, the rate of micelle production increasing as the micelle concentration increases. With each C_n species potentially initiating a new production cycle, this process represents an autopoietic unit.

Within this framework, there is no mention of DNA or proteins, but the present autopoietic system can be considered as a cell that metabolizes low-molecular-weight components. A true 'protocell' will require DNA, RNA and proteins to encode and transfer genetic information; but it provides a useful new perspective to recognize that a primitive mechanism for self-replication and metabolism can exist without them.

To what extent can our autopoietic micelle formation be truly relevant to prebiotic chemistry? Because of the simple mechanism underlying their spontaneous formation, aqueous micelles are plausible candidates for the first self-replicating bounded structures. The next question to address is to what extent such micelles may be biologically relevant. The micelles themselves are too simple, too small and dynamically too unstable to offer a plausible model for protocells, but the observation that they are, under certain conditions, precursors for vesicles, may help to forge a possible link between micelles and protocells. These larger bilayer bounded structures are better models for the prebiotic cell, as emphasized by Deamer and coworkers for liposomes¹³. Finally, we contrast the replication of the bounded structures described here with the template-based replicating structures of von Kiedrowski⁵, Rebek⁶ and their collaborators. If one draws analogies with real cells our work can be considered to represent 'shell replication', the replication of the skin of the cell. The work of von Kiedrowski and Rebek, however, is more directly related to 'core replication', namely the replication of the contents of the first cells, supposedly containing nucleic acid material. Shell and core replications are in a sense complemen-

tary views which one might eventually hope to see unified in a synthetic system. □

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Warming of the water column in the southwest Pacific Ocean

Nathaniel L. Bindoff* & John A. Church*†

* CSIRO Division of Oceanography, and

† Co-operative Research Center for Antarctic and Southern Ocean Studies, GPO Box 1538 Hobart, Tasmania 7001, Australia

THE response of the deep ocean to long-period temperature variations at the ocean surface is a crucial issue in understanding climate change¹. There are, however, very few observations available for studying changes in the thermal structure of ocean interiors. On the basis of measurements made 22 years apart of full-depth temperature sections in the Pacific Ocean between Australia and New Zealand, we show here that there has been a depth-averaged warming of 0.04 °C and 0.03 °C at 43° S and 28° S, respectively, throughout most of the water column below the mixed layer. The sea-level rise caused by expansion between a depth of 300 m and the ocean floor is 2-3 cm, consistent with the observed rate of global sea-level rise². In the main thermocline there is a coherent cooling and freshening on density surfaces, consistent with surface warming in the Southern Ocean where these waters originate. Similar observations in the North Atlantic³ show comparable changes in the thermal structure and water-mass volumes, but further measurements in other regions are required before firm conclusions can be drawn about the global significance of these changes.

The SCORPIO sections across the Pacific Ocean, nominally at 43° S and 28° S, were measured in March and July 1967 respectively⁴, using Nansen bottles. The western portions of both of these sections (between Australia and New Zealand) were measured again by the RV *Franklin* in September 1989 and March 1990. These modern high-density, eddy-resolving sections consist of 36 casts (vertical profiles of temperature and salinity) in each section, compared with 21 (28° S) and 18 (43° S) for the same portion of the SCORPIO sections. Although the number of casts is small, these sections are currently the best available in the Southern Hemisphere oceans for examining climate changes. To make quantitative comparisons and to

reduce aliasing and biases caused by the different sampling distributions, the data have been objectively mapped^{5,6} onto common geographical coordinates.

Mesoscale eddies and seasonal variations are the largest source of natural variability in these data. The root-mean-square (r.m.s.) estimate of the noise⁷ was obtained from the 1989 and 1990 repeat cruises (Fig. 1). The r.m.s. differences are smallest at a depth equivalent to 4,000 decibars pressure (0.009 °C, 0.004 practical salinity units (p.s.u.)), but greater than the accuracy of the SCORPIO⁴ and *Franklin* data (0.005 °C, 0.003 p.s.u., and 0.003 °C, 0.003 p.s.u., respectively). The objectively mapped sections resolve the mesoscale eddies and seasonal variations. The

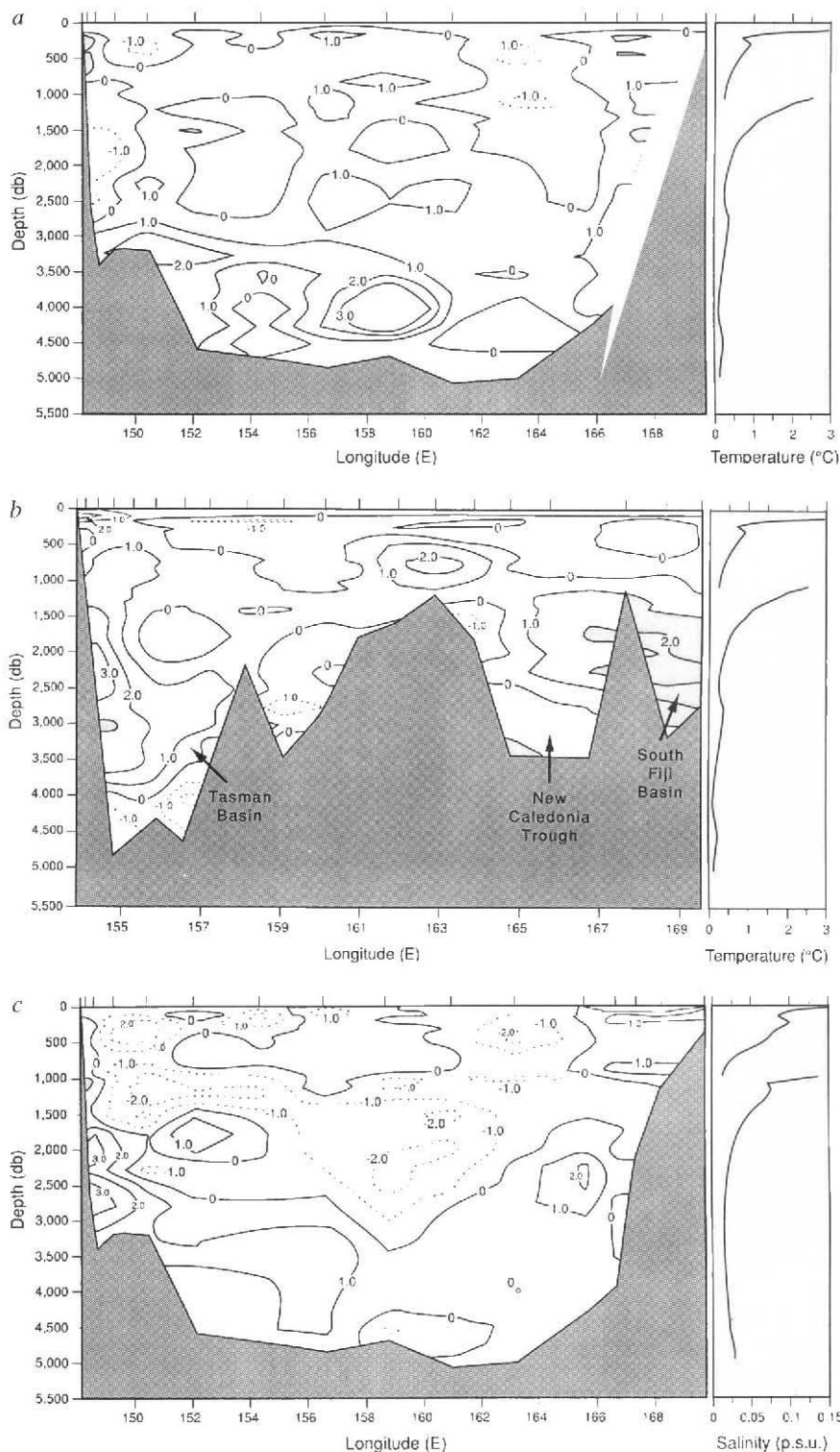


FIG. 1 The difference sections between the average of the two RV *Franklin* cruises, and the SCORPIO cruise normalized by the root-mean-square variability: *a*, potential temperature at 43° S; *b*, potential temperature at 28° S; and *c*, salinity at 43° S. The side partition is the r.m.s. variability of the potential temperature or salinity field estimated from the two cruises as half the mean-squared difference in each field at the same point in space. Below 1,000 dbar the r.m.s. variability for potential temperature and salinity has been increased by a factor of 10 and 5 respectively. Positive changes are an increase in temperature and salinity on isobars.

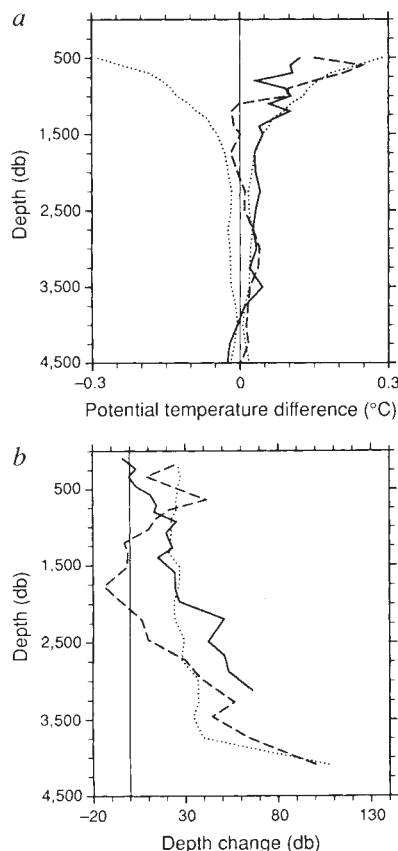


FIG. 2 Mean difference fields across each section: *a*, potential temperature on isobars, and *b*, the mean change in depth of isotherms. The dashed and continuous lines are for 43° S and 28° S respectively. The 90% confidence interval at each depth (dotted line) is based on *t*-test criteria and takes into account the number of degrees of freedom, the error in differencing the two fields, and the natural variability of temperature or depth field caused by mesoscale eddies and seasonal changes (but not interannual variability). Positive changes are a warming on isobars or an increased depth of isotherms.

difference sections were created by subtracting the objectively mapped 1967 SCORPIO data from the averaged section of the two recent *Franklin* cruises. In this analysis this is formally equivalent to finding the least-squares fit to a linear trend between each of the three realizations.

At 43° S, two coherent warming regions centred on depths of 700 dbar (5–10 °C water) just above the salinity minimum and 3,000 dbar (1.3–1.9 °C water near the salinity maximum) are present. Around 1,500 dbar there is a slight cooling. At 28° S,

TABLE 1 The areas of the water masses (defined by temperature intervals) for the SCORPIO sections and the percentage increase of these water masses in the average *Franklin* sections

Water mass	43° S Gm ²	28° S Gm ²	Per cent change 43° S	Per cent change 28° S
<1.4	1.78	0.32	-3.34	-5.7
1.4–2.4	1.85	0.90	3.53	0.77
2.4–6.0	1.74	1.24	-1.30	1.21
6.0–10.0	0.92	0.54	2.67	2.9
>10.0	0.49	0.85	-0.53	1.9

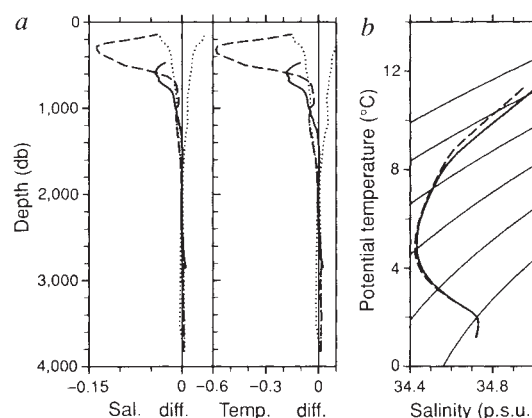
The water masses represent bottom water, salinity maximum water, salinity minimum water, thermocline waters and surface waters respectively. (Gm² are 10⁹ m²).

the pattern of change is less coherent, but there is broad-scale warming of the 1.5–5.0 °C water between the salinity minimum and maximum (1,000–3,500 dbar) in the New Caledonia Trough and the Tasman and South Fiji Basins (Fig. 1*b*). The salinity differences for 43° S (Fig. 1*c*) show freshening at 1,250 dbar of the waters between the salinity minimum and maximum (2.3–4.1 °C), and below 3,000 dbar the salinity field is more saline (<1.3 °C). Because of doubts about the quality of the deep salinity data for the 28° S section in SCORPIO (the 'anomalous' high-salinity water⁸), we present no salinity difference sections for 28° S.

When averaged along isobars, the temperature differences below the region of seasonal variation indicate that there is warming throughout most of the water column (except near the very bottom; Fig. 2). The most significant differences, relative to the 90% confidence limits, occur near 3,000 dbar with warming in excess of 0.04 °C. At 700 dbar, the 43° S section has a second peak that corresponds to a warming of 0.25 °C; this is comparable in magnitude to that observed in the North Atlantic^{3,9}. The average salinity difference field (not shown) indicates a decrease between 1,000 and 2,500 dbar and an increase below 2,500 dbar.

The warming at constant pressure is equivalent to a deepening of isotherms (Fig. 2*b*). At 43° S, the 2.4 °C isotherm (2,000 dbar) has not changed depth, but isotherms above and below this have deepened by up to 45 and 90 dbar respectively. The nonuniform deepening of isotherms results in a reduction in the thickness of the salinity minimum (2.4 to 6 °C) waters (Table 1). The distance between the 1.1 and 2.4 °C isotherms has increased by 90 dbar, leading to a 3% increase in salinity maximum water and a corresponding reduction in Antarctic Bottom Water. At 28° S, the depth of all isotherms has increased (except at the bottom), implying that all water masses present in this section, except bottom water, have increased in volume. The volume changes, implied by the change in thickness of the layers at 43° S and geometry of the Tasman Basin, over the 22 years

FIG. 3 *a*, Sectional averages of the change of salinity (Sal. diff.) and potential temperature (Temp. diff.) on neutral surfaces (plotted as a function of the mean depth of each neutral surface). The dashed and continuous lines are for 43° and 28° S. The dotted line is the 90% confidence interval on these surfaces as described in Fig. 2. Negative changes are a decrease in salinity and temperature between 1967 and 1990. *b*, The mean potential temperature–salinity diagram for the 43° S section in 1967 (continuous line) and 1990 (dashed line). The six curved lines are potential density surfaces (σ_θ values of 26.5, 26.75, ..., 27.75).



require a transport change of ~ 0.2 Sverdrup. These temperature and salinity changes are not density-compensating and steric sea level has changed. The steric sea level, integral of specific volume anomaly from the bottom to 300 dbar, has increased by 2 and 3 cm at 43° S and 28° S respectively, and is consistent with the estimates of global sea-level rise of 1.0–3.0 cm per decade².

A highly significant freshening (and cooling) on neutral surfaces¹⁰ (almost equivalent to potential density surfaces in this case) in the main thermocline (Fig. 3) coupled with a warming on isobars was found at 43° S and 28° S. The peaks in the salinity changes in each section occur on almost the same neutral surface. A simple model of the ocean's response to atmospheric warming is a warming of the surface waters at constant salinity and subsequent subduction of these waters along neutral surfaces into the ocean interior. As a result, the main thermocline waters become warmer on isobars but, surprisingly, they become cooler and fresher on a density surface¹¹, as observed (Fig. 3b).

The pattern of change is consistent with Tasman Sea circulation. Because of the shallow topography to the north and east of 28° S, bottom and salinity maximum waters can only enter the Tasman Sea from the south, and both sections show an

increase of salinity maximum water at the expense of bottom waters. In contrast to the deep waters, upper thermocline water (including Antarctic intermediate water) enters the Tasman Sea both from the south (across 43° S) and, after circulating around the subtropical gyre¹², from the north (across 28° S). The temperature change on isobars and salinity change on the same neutral surfaces in the thermocline are largest at 43° S, which is consistent with these water masses being more recently ventilated by contact with a warmer atmosphere than the 28° S waters.

Although model results^{1,13,14} suggest that climate change is largest in the main thermocline, observation of this warming will in general be masked by eddy and seasonal variability. But, as demonstrated, longer-term changes can be more easily diagnosed by examining water mass characteristics on neutral surfaces. Below the thermocline, the signal-to-noise ratio is larger on isobars and significant warming is observed. Repeat experiments such as these have an important role in testing predictions and initialization of global ocean-atmosphere models of climate change. In assessing greenhouse climate change, similar repeat observations should be completed for the Indian and south-eastern Pacific and Atlantic Oceans. □

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Molecular records of twentieth-century El Niño events in laminated sediments from the Santa Barbara basin

Julie A. Kennedy & Simon C. Brassell*

Department of Geology, Stanford University, Stanford, California 94305-2115, USA

ORGANIC molecules preserved in sediments, especially long-chain alkenones, can preserve and record evidence of past climate variations^{1–5}. A dependence of alkenone distributions on temperature has been recognized in cultures of the unicellular coccolithophorid alga *Emiliania huxleyi*^{1,6}, which survives in sediments and water-column particulates⁶. Through this dependence, stratigraphic profiles of the degree of alkenone unsaturation (expressed as U_{37}^K values¹) have been shown in several oceanic settings to reflect long-term changes in sea surface temperatures associated with glacial-interglacial cycles^{1–5}. Here we show that annual variations in alkenone unsaturation in laminated sediments from the Santa Barbara basin, off the shore of California, reveal increases in water temperatures which can be linked directly to the observed sequence of El Niño events in the twentieth century⁷. The range and variations in water temperatures, calculated from U_{37}^K values (12.8–15.2 °C) using the relationship established from culture experiments^{6,8}, broadly correspond to the annually averaged temperatures (11.3–15.2 °C) measured intermittently by the CalCOFI programme at 20 m depth between 1953 and 1984. These results show that, in favourable settings, alkenone unsaturation profiles can provide an annually resolved record of oceanic temperature

changes, and may constitute a valuable tool for describing and evaluating the history of global climate variability and change.

Molecular stratigraphy details the sedimentary record of fluctuations in the abundance of specific organic molecules and classes of organic compounds over time. Patterns of variability in these compounds can be used to interpret the history of dynamic change in the populations of the biota from which they were derived. Among marine phytoplankton, such change is frequently driven by and coincident with climate oscillations and can be recorded in the stratigraphic profiles of molecules with chemical structures that are diagnostic indicators of contributions from particular organisms^{9–11}. One such group of molecules, alkenones, has been investigated in several recent palaeoclimatic studies^{1–3,12,13}. These compounds are a series of di-, tri- and tetra-unsaturated long-chain ketones^{14–16} produced by a restricted number of species of prymnesiophyte algae, most notably the ubiquitous coccolithophorid alga *E. huxleyi*^{17,18}. This eurythermal organism is viable over a wide temperature range (2°–29 °C; ref. 19) apparently through biosynthetic regulation of the degree of unsaturation in these compounds, increasing its production of more unsaturated alkenones as water temperature decreases^{1,6,8}. The relationship between alkenone unsaturation and growth temperature, expressed as the unsaturation index U_{37}^K , has been demonstrated and calibrated in studies of natural populations and laboratory cultures^{1,6,8}, yielding an equation which permits conversion of U_{37}^K values to absolute growth temperature^{6,8}. The unique temperature record provided by the alkenones, the survival of that signal in the sedimentary record²⁰ and the longevity of biosynthetic production of alkenones shown in the geological record²¹ has allowed them to be successfully exploited in studies of modern and ancient climatic variability^{1–3,12,13}.

Here we demonstrate the use of alkenones in high-resolution palaeoclimatic investigations addressing variations associated with El Niño events, a study helped by the analytical capability to determine alkenones in small sediment samples (about 6 g wet weight). Specifically, laminated sediments from the Santa

* Present address: Biogeochemical Laboratories, Department of Geological Sciences, Indiana University, Bloomington, Indiana 47405-5101, USA.

Barbara basin, offshore southern California, reveal alkenone profiles that accurately record historically documented patterns of year-to-year sea surface temperature (SST) variations driven by the El Niño climate phenomenon. The ability of alkenone stratigraphic studies to characterize this specific climatic regime holds particular promise as the influences of El Niño, manifest in a warming of SST, occur throughout much of the Pacific basin.

The Santa Barbara basin (SBB) was chosen as an optimum site for the detailed investigation of stratigraphic variation during the twentieth century. Waters derived from the local oxygen minimum zone spill over the sill (475 m) of the bowl-shaped basin and exert a strong control on the oxygen content of the deepest waters (to about 590 m). Further oxygen depletion results from oxidation of the organic matter raining down from the highly productive overlying surface waters²² and leads to dysaerobic to anaerobic conditions ($<0.1 \text{ ml l}^{-1} \text{ O}_2$) in the centre of the basin. Such low oxygen waters cannot support populations of benthic burrowing and grazing organisms, so the bottom sediments are not disturbed by bioturbation^{23,24}. The result of these conditions is the deposition of an organic-rich annual couplet of a light and a dark sediment layer which contain information on the physical, chemical and biological characteristics of basin waters. The preservation in SBB of annually laminated or varved sediments over thousands of years has made it the focus of many palaeoclimatic and palaeoceanographic investigations²⁵⁻²⁹. SBB sediments also offer the advantage of cross-correlation of cores from within the central basin which

have been collected at various times from different sites. These relationships can be made because individual laminae contain characteristic pore fluid contents, permitting core-to-core correlation based on this parameter³⁰.

Three sediment subcores (8.3 cm internal diameter; designated 9, 10a, 10b) were taken from box cores collected in the anoxic centre of the SBB ($34^\circ 14' \text{ N}$; $120^\circ 01' \text{ W}$) during a sampling cruise aboard the *RV Sproul* operated by Scripps Institution of Oceanography (SIO) in February 1988. Individual cores were held in cold storage for sampling later. All were sampled on a laminae by laminae basis (2–4 mm) using visual guides of sediment colour change. Subsamples from two of the cores (10a, b) were stored frozen and then analysed using standard techniques for sonic extraction of wet sediments. Those from core 9 were weighed, freeze-dried, reweighed for calculation of pore fluid content and extracted as dry sediments. Calculation of their pore fluid contents allowed direct correlation of all cores with those dated according to the stratigraphy established by the extensive work of investigators at SIO³⁰. Comparison of the records based on pore fluid content data to the subsurface depths of specific marker layers (1984, 1959 and 1919) showed agreement to within about 5 mm. Thus, the accuracy of the timescale for the cores is concluded to be within the same range as that reported for SIO cores (uncertainty $\pm 1 \text{ yr}$).

The U_{37}^K values determined for the cores range from 0.475 to 0.553 and exhibit a series of peaks and troughs in their profiles (Fig. 1a). There is a good correspondence in the values for duplicate analyses on the upper sediment portion for two cores

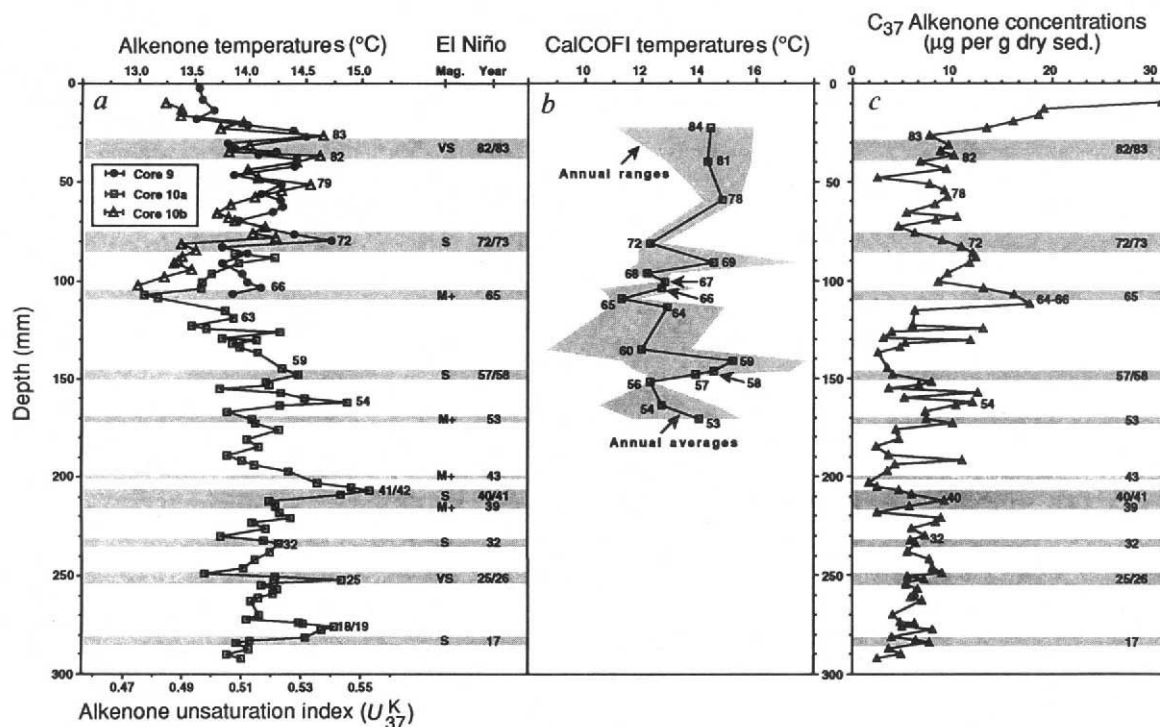


FIG. 1 a, Profile of twentieth-century variability in alkenone temperatures derived using the equation⁸ $U_{37}^K = 0.0347 + 0.039$ from the alkenone unsaturation indices (U_{37}^K values) for three cores of laminated sediments from the Santa Barbara basin. The key to the different cores also shows the margin of error (± 0.003) in determination of the U_{37}^K values, which corresponds to an error in temperature determinations of $\pm 0.09^\circ \text{C}$. Details of sediment extraction, TLC fractionation of lipid extracts, sulphur removal, and the procedures for alkenone analysis by gas chromatography, confirmation of their identification by gas chromatography/mass spectrometry and their quantitation are given elsewhere³¹. The age assignments for specific depths are based on the stratigraphy for the SBB established at SIO³⁰. Magnitudes and dates of equatorial El Niño events are shown with their intensities assessed as 'very strong' (VS), 'strong' (S) and 'moderate to

strong' (M+)⁷ and with their rough duration represented by the shaded intervals. Note the general agreement between warm excursions in the molecular temperature record and El Niño events, allowing for the 1-year time lag in their appearance offshore California relative to their manifestation in equatorial regions. b, Annual averages of all CalCOFI temperatures (irrespective of season) measured at 20 m water depth plotted against age equivalent sediment depths. The shaded area represents the range of values recorded in each given year. c, Downhole profile of C_{37} alkenone concentrations showing major fluctuations in their abundance which do not correspond with El Niño events (shaded as in a). The significance of the productivity signals recorded in the concentrations of alkenones and various sterols, their comparative resilience to diagenetic alteration and their links to observed plankton blooms (for example red tides) are discussed elsewhere³¹.

(9 and 10b) collected from adjacent sampling sites. The lower part of the curve is derived from analyses of samples from core 10a, a duplicate core collected from the same box core as 10b. The U_{37}^K values were used to calculate absolute temperatures (Fig. 1a) according to the revised equation established for culture experiments and validated for oceanic particulate matter^{6,8}. Given the variable time lag between the equatorial and the California El Niño (~1 yr) and the aforementioned stratigraphic uncertainty, it is evident that each major ENSO (El Niño/Southern Oscillation) event⁷ occurring during the interval examined is represented by a positive excursion in SST values deduced from the alkenone profiles (Fig. 1a). Most of the enhancements in alkenone temperature correspond to ~1 °C and are either concurrent with, or immediately postdate, each El Niño. The results demonstrate a clear link between the historical record of 'strong' and 'very strong' ENSO events⁷ and SST warming recorded in sedimentary alkenone profiles; the correspondence with 'moderate to strong' events is less apparent. But the magnitude of individual excursions does not directly reflect the rating scale⁷. For example, warming associated with the 'very strong' 1982/83 El Niño event was measured at 3–4 °C in the spring of 1983^{32,33}, and is here represented by a warming signal of much lower magnitude (~1 °C). Such discrepancies between observed temperature shifts and values calculated from molecular signals should not be unexpected. First, the sampling intervals are close to the annual accumulation rate (3–4 mm) and the U_{37}^K temperatures reflect a yearly average of upwelling and non-upwelling signals and the concomitant annual variations in alkenone productivity. Second, the observed record may also reflect fluctuations in the seasonal timing or depth of maximum alkenone production induced by changes in physical or chemical oceanography (for example nutrient supply, SST) associated with an El Niño event of a given magnitude. Third, there may be differences in the magnitude of individual events between the equatorial and offshore California regions. Interestingly, comparison of alkenone data with those from Peru¹³ reveals that evidence of El Niño events seems better preserved in molecular records far from the area of maximum ocean warming. In offshore Peru this climate change greatly reduces phytoplankton productivity, thereby greatly attenuating the intensity of the warm signal through dilution in the sedimentary record.

The SST calculated from alkenone distributions generally agree with those measured at 20 m water depth in the SBB by the California Cooperative Oceanic Fisheries Investigations (CalCOFI) during the period 1953 to 1984 (Fig. 1b). The temperature ranges (12.8–15.2 °C for U_{37}^K ; 11.3–15.2 °C for CalCOFI annual averages) are consistent and the nature and frequency of their variations are comparable. Direct comparison between

the two data sets is precluded by the intermittent nature of the CalCOFI record where the sequence of annual sampling is irregular (no data were collected for 15 out of the 32 years) and lacks seasonal consistency³¹. Also, two aspects of the selection and treatment of CalCOFI data warrant discussion. First, examination of plankton biomass at multiple stations offshore southern California revealed that the coccolithophorid *E. huxleyi* was associated with the chlorophyll maximum at ~22 m water depth at a temperature of 13 °C (ref. 34). Hence, the temperature comparisons are based on CalCOFI measurements at 20 m, which is deemed equivalent to the depth of predominant coccolithophorid, and hence alkenone, production. Second, the CalCOFI data are presented as annual averages (individual temperatures range from 8.71–17.66 °C at 20 m; Fig. 1b) to match the annually accumulated signals reflected in the U_{37}^K values for the sediments. Such treatment seems appropriate given that some coccolithophorids seem to be less dependent on seasonal upwelling and nutrient supply than other plankton groups. For example, *E. huxleyi* has a competitive advantage over the diatom *Skeletonema costatum* where nitrate levels were <0.5 µM (ref. 35). The capacity to thrive at lower nutrient levels was attributed to their use of low levels of ammonium ions as a nitrogen source, an ability which may enable them to prosper in environments with limited nutrient supply and which should eliminate their dependence on replenishment of nitrate by upwelling. Given these characteristics of coccolithophorid production it is not surprising that the downcore variations in alkenone concentrations (Fig. 1c), which range from 1.7 to 30.9 µg C₃₇ alkenones per g dry sediment, show no apparent correspondence to El Niño events³¹.

Overall, this investigation demonstrates that molecular profiles in sediment sequences compare well with observational data and can record historically documented changes in SST. Such signals seem well preserved in laminated sediments, as in the SBB, and merit further investigation to assess their value as measures of short-term climate change on a global scale. The alkenone distributions clearly reflect climate fluctuations driven by El Niño, the oceanic component of the ENSO phenomenon, which represents the predominant signal in year-to-year climate variability and is a key to the broader understanding of the coupled ocean-atmosphere climate system³⁶. The detailed patterns (magnitude, rates and geographical scale) of ENSO events in more ancient sequences may provide evidence of such characteristics before the introduction of atmospheric contaminants which may be noticeably altering the present climate of the Earth and its future course. Thus, differences between such patterns of modern and pre-industrial El Niño events may help to clarify anthropogenic influences on climate variability linked to global warming. □

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Solubility of neutral nickel in silicate melts and implications for the Earth's siderophile element budget

Russell O. Colson

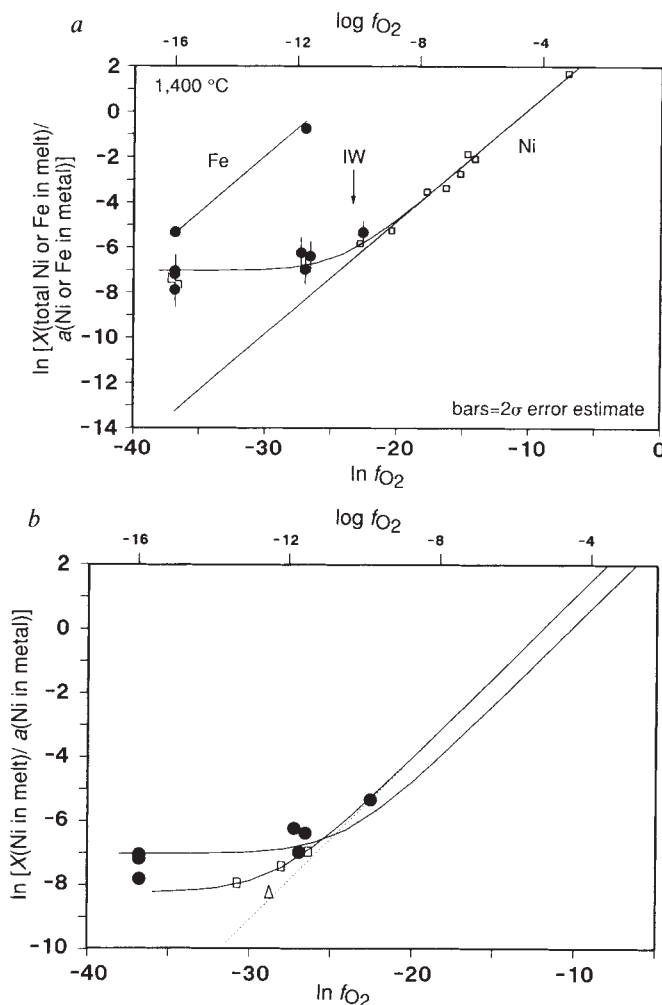
Department of Earth and Planetary Sciences and McDonnell Center for the Space Sciences, Washington University, St Louis, Missouri 63130, USA

EXPERIMENTAL studies have so far suggested that an insignificant amount of neutral nickel (Ni^0) is soluble in silicate melts¹⁻⁴. This has led to difficulties in explaining the high concentrations of nickel in the modern Earth's mantle, because virtually all nickel (along with other siderophile elements such as cobalt and the noble metals) would have been removed from the mantle when the Earth's metallic core separated from it at low oxygen fugacity^{1,2,5}. Several models have been proposed to explain the Earth's siderophile element budget^{1,6-8}, each based on the

belief that the solubility of neutral metal species in silicate melts is negligible. Here, however, I present experimental evidence indicating that the solubility of at least one neutral siderophile element, nickel, is not negligible. Because the presence of Ni^0 will affect the partitioning of nickel between silicate melt and metal, and between silicate melt and crystalline silicate phases, these results have implications for our understanding of petrogenetic processes that take place in conditions of low oxygen fugacity, where Ni^0 is an important part of total nickel. In particular, the Ni^0 solubilities found in the present study are large enough to explain the anomalously high concentrations of nickel in the Earth's mantle if the temperature of the early mantle was sufficiently high ($\geq 1,800^\circ\text{C}$).

I equilibrated silicate melt (composition SiO_2 54.4 wt%, Al_2O_3 19.3%, CaO 9.0%, MgO 15.9%) with nickel or nickel-metal alloys in a Deltech gas-mixing furnace at selected values of oxygen fugacity (f_{O_2} between 10^{-10} and 10^{-16}) and temperatures (1,400, 1,500 and $1,560^\circ\text{C}$) for times ranging from 12 minutes to over 16 hours. The silicate melt was quenched to glass and concentration of total nickel (Ni^{2+} plus Ni^0) in the glass next to the metal was determined by electron microprobe analyses. The relative contributions of Ni^{2+} and Ni^0 to total nickel in the glass were determined by evaluating how the total nickel in the

FIG. 1 *a*, Variation of total nickel in silicate melt as a function of f_{O_2} . ●, this study; ■, results from ref. 10. The straight line indicates the trend expected if nearly all nickel were present as Ni^{2+} ; the curve is a fit of equation (5) to these data (solving for K' and K'' at $1,400^\circ\text{C}$). At f_{O_2} values below the iron-wüstite buffer (IW), nickel concentrations in the melt become independent of f_{O_2} , indicating that Ni^0 then becomes the dominant nickel species in the melt. Values of f_{O_2} were controlled by CO_2 -CO mixtures (f_{O_2} near 10^{-12}) or by one of the buffers CO-graphite (f_{O_2} near 10^{-16}) or Mo-MoO₂ (f_{O_2} near 10^{-10}), and were calculated from known free energies of formation for CO_2 , CO, C, Mo and MoO₂. Samples were suspended on platinum loops for experiments using CO_2 -CO mixtures or were contained in graphite or molybdenum capsules. Experiments in graphite or Mo capsules were done in CO or argon atmospheres, respectively. Nickel generally formed an alloy with the Pt loops, graphite or molybdenum capsules, or iron metal (iron was present in one experiment at $\sim 10^{-12} f_{\text{O}_2}$). I therefore calculated the activity of nickel from ref. 24 on the basis of metal compositions determined by electron microprobe. Concentrations of nickel ($\text{Ni}^0 + \text{Ni}^{2+}$) in the silicate melt were determined by electron microprobe using high current (~ 150 nA) and methods outlined in ref. 25 to obtain sensitivities down to 10 p.p.m. In some cases, nearby Ni-rich metal was masked with Ag conducting paint to prevent stray electrons from the microprobe beam from exciting nickel in the metal and causing anomalously high apparent concentrations of nickel in the glass. For other samples, analyses were corrected for interference from nearby Ni-rich metal estimated numerically as $B \times \sum [A(x)/x^2]$, where $A(x)$ was the area of exposed Ni metal at distance x from the analysis point (x taken in 10- μm increments) and B a coefficient determined from the analyses of a 'blank' (brass metal) at various distances from a Ni metal standard. Both methods were used for one experiment, at f_{O_2} near 10^{-12} , and the determined concentrations were identical within uncertainty. Typically, concentrations were extrapolated to the melt-metal interface as explained in Fig. 3. Two analogous experiments were done with iron in place of nickel. Reduction potentials for Fe^{2+} are lower than for Ni^{2+} (ref. 26), so Fe^0 should not become the dominant iron species until f_{O_2} is much lower than the value at which Ni^0 becomes dominant. If experimental complications or changes in melt structure with f_{O_2} (rather than presence of Ni^0) are causing the nickel concentrations in the melt to seem independent of f_{O_2} , then Fe concentrations should behave like Ni concentrations at a similar f_{O_2} . The results suggest that Ni^0 is indeed the dominant species at low f_{O_2} . The Fe-bearing experiments were also used as an independent check of f_{O_2} . The line drawn through the Fe data represents the concentrations of Fe^{2+} expected at the corresponding f_{O_2} as predicted from independent data²⁶. Oxygen fugacities calculated from the Fe-Fe²⁺ buffer are identical within uncertainty to those calculated from the graphite-CO buffer and the CO_2 -CO gas buffer. *b*, Data from refs 1 (Δ) and 3 ($\square$) at $1,270^\circ\text{C}$ and $1,300^\circ\text{C}$ respectively, compared with my data. Concentrations of nickel in melt are higher than expected if all nickel were present as Ni^{2+} (dashed line calculated from the reduction potentials of ref. 10 using Ni^{2+} activity coefficients estimated from ref. 27). In addition, the data of ref. 3 have a slope distinctly different from that expected if only Ni^{2+} were present. Schmitt *et al.*³ explain the slope as being due to a decrease in the Ni^{2+}

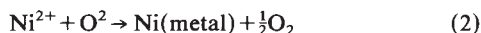


activity coefficient as Fe^{2+} is removed from the melt with decreasing f_{O_2} . But this variation of Ni^{2+} activity coefficient with changing melt composition is opposite to that expected²⁷. My experiments were done in the absence of Fe, yet a similar trend is still observed; A more reasonable explanation is the increasing importance of Ni^0 at lower f_{O_2} . The data of ref. 3 can be fitted successfully to equation (5), where $K'_{1,300}$ (and thus Ni^{2+} concentration as a function of f_{O_2}) is determined from independent data^{10,27} and $K''_{1,300}$ (and thus Ni^0 concentration) by regression to equation (5).

melt changes with f_{O_2} , as explained below. The concentration of Ni^{2+} , $X(Ni^{2+})$ with f_{O_2} varies according to the relationship

$$X(Ni^{2+}) = [(f_{O_2})^{1/2} a_{Ni\ metal}]/K' \quad (1)$$

where a is activity, derived for the reaction



K' is constant at constant temperature, pressure and melt composition where O^{2-} activity is buffered at a constant value⁹. The concentration of Ni^0 in the melt is independent of f_{O_2} and is given by

$$X(Ni^0) = (a_{Ni\ metal})/K'' \quad (3)$$

From equations (1) and (3), it is apparent that at sufficiently high f_{O_2} , most nickel ($Ni^0 + Ni^{2+}$) in the silicate melt will be Ni^{2+} , and at fixed temperature and Ni metal activity the solubility of nickel will vary with f_{O_2} roughly according to the relationship

$$\ln X(\text{nickel in melt}) = \frac{1}{2} \ln f_{O_2} + \text{constant} \quad (4)$$

At sufficiently low f_{O_2} , the concentration of Ni^{2+} will become insignificantly small, most of the nickel in the melt will be Ni^0 and the solubility of nickel will become independent of f_{O_2} .

The concentration of total nickel in the melt can be expressed algebraically as a function of f_{O_2} by the relationship

$$\ln (X_{Ni^0} + X_{Ni^{2+}})_{\text{melt}} = -\ln(K') + \ln(f_{O_2}^{1/2} + K'/K'') \\ + \ln(a_{Ni\ metal}) \quad (5)$$

which is similar to that derived in ref. 10.

Experimental results at 1,400 °C for several values of f_{O_2} are shown in Fig. 1a. The curvature of the data, as predicted by equation (5), demonstrates that, at 1,400 °C, Ni^0 becomes an important nickel species at an oxygen fugacity slightly above that of the iron-wüstite buffer. Solubility of Ni^0 in melt in equilibrium with Ni metal is determined to be ~0.103 wt% (± 0.03) at 1,400 °C in this composition.

Indications of Ni^0 solubility have been found in previous experimental work³ but were interpreted as being due to a strong dependence of Ni^{2+} activity on Fe concentration in the melt.

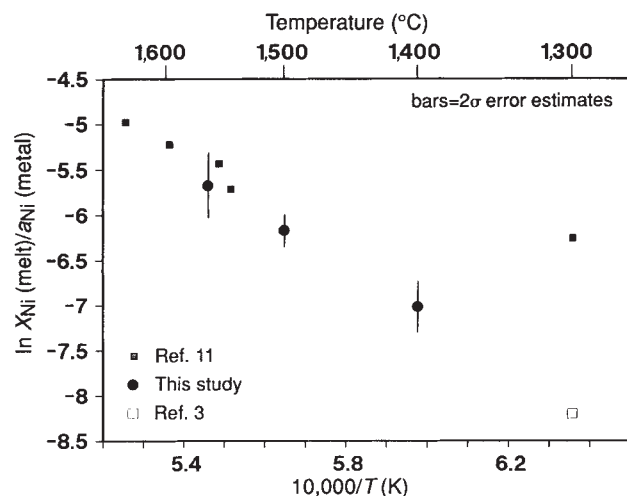


FIG. 2 Dependence of Ni^0 solubility on temperature. The value at 1,400 °C is determined by solving for K' and K'' in equation (5) for the data shown in Fig. 1a. The value at 1,500 °C is the average of two experiments (Fig. 3b) done in graphite capsules in CO ($\log f_{O_2} = -15.8$). That at 1,560 °C is from a single experiment in a graphite capsule in CO ($\log f_{O_2} = -15.4$). The value for Schmitt *et al.*³ is determined as described in Fig. 1b. Values from ref. 11 are determined by subtracting a calculated amount of Ni^{2+} (refs 10, 27) from the total nickel reported; calculated values of Ni^0 are therefore very uncertain at the lowest temperature (~1,300 °C) where the ratio of Ni^0 to total nickel is low, and the deviation of the point at 1,300 °C from the general trend is not believed to be significant.

Figure 1b demonstrates that those results are better explained by Ni^0 solubility. The solubility of Ni^0 at 1,300 °C is determined from the experiments of ref. 3 (as explained in Fig. 1b) to be 0.032 wt%, indicating a strong dependence of Ni^0 solubility on temperature. This temperature dependence has been confirmed by additional experiments at 1,500 and 1,560 °C, and the results are shown in Fig. 2 together with Ni^0 solubilities calculated from ref. 11. These data, excluding the single point from ref. 11 at 1,300 °C, yield the following relationship

$$-\ln K'' = \ln [X(Ni^0, \text{melt})/a_{Ni\ metal}] \\ = 10.6(\pm 0.12) - 29,461(\pm 1,200)/T \quad (6)$$

where temperature T is in kelvin and errors are $\pm 1\sigma$. This heat of mixing is comparable to that for Pb^0 dissolved in silicate melt¹².

Although the fit of the data to equation (5) (Fig. 1a, b) and the consistent variation of apparent Ni^0 concentration with temperature (Fig. 2) suggest that Ni^0 is in true solution in these silicate melts, the presence of suspended nickel metal particles in the melt has complicated previous attempts to measure metal solubilities in the melt³, and further demonstration of an approach to equilibrium is warranted. The approach to equilibrium from both high and low initial concentrations of nickel in the melt is shown in Fig. 3a and b. These bracketing experiments illustrate that Ni^0 approaches either thermodynamic equilibrium or some steady-state concentration very like equilibrium.

If apparent Ni^0 solubility is related to metal in suspension in the melt, and the amount of metal in suspension is somehow regulated at a 'steady state', Ni^0 in the melt should vary linearly with the concentration of nickel in the metal with which the melt is equilibrated. In contrast, if Ni^0 is in true solution in the melt, its concentration should vary with the activity (not the concentration) of Ni in the metal. To distinguish between these possibilities, I equilibrated silicate melts with various Ni -metal alloys for which the concentration-activity relationships are distinctly different, thus allowing activity and metal composition to be varied roughly independently. The results (Fig. 4) demonstrate that the concentration of Ni^0 in the melt is correlated to activity of Ni in the metal, not its concentration, supporting the belief that the Ni^0 is in true solution.

I also investigated whether small metal particles were present in the melt by using backscattered electron imaging. I used a high current (~150 nA) to increase the contrast between high-density metal particles and melt so that metal beads down to at least 0.1 μm could be resolved. For most of the analyses reported here, no nearby metal beads were seen, although submicrometre metal beads were seen in some regions of some experiments. The presence and size of these beads were poorly correlated to nickel concentrations in the melt, suggesting that they are not the primary source of the nickel measured. Their presence and size seem to be related to the quench rate of the experiment, suggesting that at least some of the beads may have formed by exsolution during the quench. In any case, their presence or absence is not a clear indicator of whether the measured nickel is in solution or suspension, because the beads seen could have formed during quenching, or more ubiquitous suspended metal might be too small to be resolved by backscattered electron imaging.

A nickel species other than Ni^{2+} associated with oxygen must be in true solution in silicate melts at low f_{O_2} . Because most of these experiments were done in the presence of carbon monoxide and carbon, there is the possibility that the soluble species include a carbonyl or carbide of nickel (in addition to Ni^0). In some experiments, however, the activity of C was very low (Fig. 1a) and there was no indication that the concentration of Ni^0 was decreased, suggesting that carbide species, if present at all, are not the dominant nickel species. In one experiment in which the activity of CO was very low (Fig. 4), the concentration of

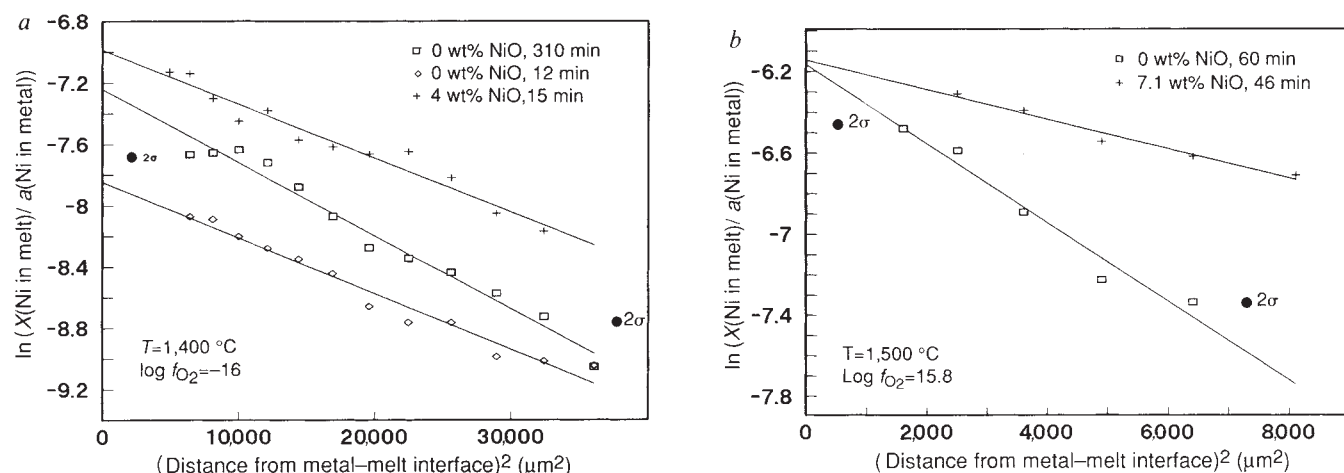


FIG. 3 *a, b*, To bracket the equilibrium concentration of Ni^0 , melts with nickel concentrations initially either much higher or much lower than the expected equilibrium concentration were equilibrated with Ni metal. Initial concentrations of NiO in the glass and durations of the experiments are indicated. The approach to equilibrium from both low and high concentrations suggests that the values reported here represent equilibrium concentrations of Ni^0 in true solution in the melt (rather than, for example, in suspension as fine particles⁴). Because of continuous loss of Ni from the experiments, apparently as a volatile species, equilibrium is only approached in the vicinity of the Ni metal (such loss of transition metals is a well known problem at low f_{O_2} in CO-rich systems and is believed to be due to formation of volatile carbonyls (ref. 28, and D. Mittlefehldt, personal communication). Equilibrium concentrations are derived by extrapolating to the metal-melt interface, in this case using a simple $\ln(C) = Ax^2 + B$ relationship. The closest analyses are $\sim 40\text{--}50\ \mu\text{m}$ from the metal. Closer than this, the correction for interfer-

ence from stray X-rays from nearby metal becomes large (see Fig. 1) and good analyses were not possible. The slopes of these profiles are related to diffusion rates, rate of volatile Ni loss, size of sample, nondiffusional mixing (such as thermal convection and, where NiO is in the initial melt, convection caused by settling of exsolved Ni metal), and orientation of the analysed profile relative to Ni metal and the edge of the glass sample. Because the configuration of the samples and the orientation of profiles differed between experiments, no simple relationship between slope of the profile and diffusion rate can be derived. Error bars represents analytical uncertainty only. But note that (for example) the profile of the 310-s experiment deviates from linearity by more than analytical uncertainty. Extrapolating (for example) from only the three points nearest the metal for the 310-s experiment results in a different value for Ni^0 solubility. This additional component of uncertainty in the extrapolated value is reflected in the error bars shown in the other figures.

nickel in the melt was $\sim 0.045\ \text{wt\%}$, lower than that expected on the basis of experiments in the presence of both C and CO. So all the nickel solubility cannot be due to solubility of Ni carbonyls, but the possibility that some Ni carbonyls are soluble cannot be ruled out.

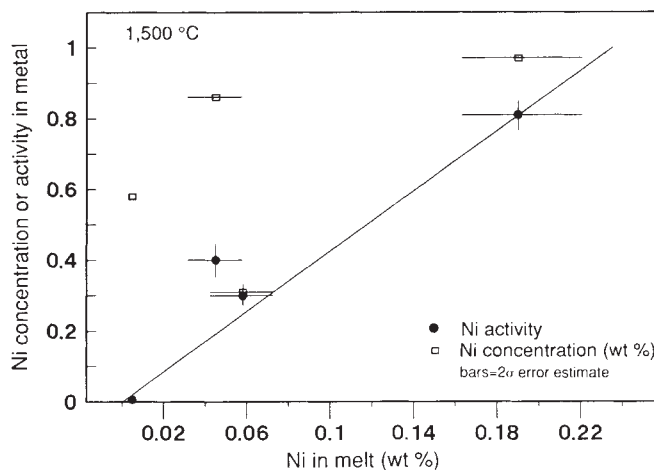
Several models exist that attempt to explain the concentrations of siderophile elements in the Earth's mantle^{1,6}. None of these completely explains all siderophile element concentrations⁶. On the basis of the previous assumption that Ni^0 solubility is negligible, however, we can state some general constraints. If the mantle was stripped of siderophile elements during core formation⁷, then an event such as addition of siderophile elements from a large impact object after core formation is required to explain the relatively high concentration of siderophile elements in the modern mantle^{6,7}. Alternatively, if the mantle was not stripped of siderophile elements, then either the mantle must

have equilibrated with some phase other than metal (such as sulphides⁸), or the separation of metal from the mantle must have been incomplete¹, a possibility that is inconsistent with a global magma ocean from which metal continuously segregated¹³.

My results indicate, however, that Ni^0 contributes substantially to total nickel solubility at the low- f_{O_2} and high-temperature conditions that probably prevailed in the early mantle¹ and potentially explains all of the nickel present in the modern mantle. If we postulate an ancient mantle temperature of $1,800\ ^\circ\text{C}$, then the concentration of nickel in equilibrium with Fe-Ni metal with Ni activity equal to 0.05 is $1,540\ \text{p.p.m.}$ ($X(\text{Ni}^0, \text{melt}) = 0.0014$). This is close to the observed current mantle composition ($2,110\ \text{p.p.m. Ni}$; ref. 14) and to calculated compositions (pyrolite with $1,694\ \text{p.p.m. Ni}$; ref. 15).

The implication of Ni^0 solubility is that much more nickel

FIG. 4 Correlation between weight per cent of nickel in melt, and activity or concentration of Ni in the metal with which the melt is equilibrated. Each experiment is represented by two points, one for Ni activity and one for Ni concentration in the metal. The points at $\sim 0.19\ \text{wt\% Ni}$ in the melt represent the mean of the two experiments at $1,500\ ^\circ\text{C}$ described in Figs 2 and 3b. The experiment at $\sim 0.058\ \text{wt\% Ni}$ is done in a graphite capsule in CO, equilibrating melt with a Pt-Ni alloy. The experiment at $\sim 0.045\ \text{wt\% Ni}$ is in a graphite capsule in argon, equilibrating melt with a Si-Ni alloy (f_{O_2} is buffered at values below the C-CO buffer by the presence of both Si and SiO_2). The experiment at $\sim 0.005\ \text{wt\% Ni}$ is in an alumina crucible in argon, equilibrating melt with a Si-Ni alloy at $1,460\ ^\circ\text{C}$. Activity of Ni in the Pt-Ni alloy was calculated from ref. 24. Activities in the Si-Ni alloys were determined from ref. 29. The straight line passing through the origin shows a linear relationship between Ni activity and Ni concentration in the melt. Although the point at $\sim 0.045\ \text{wt\% Ni}$ falls above this line, the correlation between activity and concentration in the melt is good. No correlation is observed between the concentration in the metal and in the melt, indicating that suspension of metal beads in the melt cannot explain the observed concentrations of Ni in melt.



was probably present in the 'depleted' mantle than heretofore expected. High Ni^0 solubility is consistent with the idea that the Earth could have had a magma ocean in its early history from which the metal core continuously separated^{13,16} and undermines the argument that a large impact object must have struck the Earth after core-mantle separation^{7,17}. This explanation does not prove that models invoking incomplete separation of metal from the mantle, or addition of siderophile elements to the mantle by meteorites or other impactors, are wrong, but such models should be re-evaluated to take into account nickel in

the neutral state. It is likely that other siderophile metals are also soluble in the neutral state in silicate melts (Cr^0 , Fe^0 , Cu^0 , Pb^0 , Ag^0 and Au^0 are reported to be soluble in silicate melts^{12,18-20}). Solubilities of uncharged siderophile elements must be determined before reasonable mass-balance calculations of these elements in the Earth's mantle are possible. Solubility of Ni^0 in silicate melts has implications for other aspects of geochemistry: it is high enough for partitioning of nickel between crystalline phases and melt, important in the evolution of magmas, to occur at low f_{O_2} or high temperature²¹⁻²³. □

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Direct radiocarbon dates for prehistoric paintings at the Altamira, El Castillo and Niaux caves

H. Valladas*, H. Cachier*, P. Maurice*,
F. Bernado de Quiros†, J. Clottes‡,
V. Cabrera Valdés§, P. Uzquiano||
& M. Arnold*

* Centre des Faibles Radioactivités, Laboratoire mixte CNRS-CEA, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

† Area de Prehistoria, Universidad de León, 24071 León, Spain

‡ Ministère de la Culture et de la Communication, 11 rue du Fourcat, 09000 Foix, France

§ Departamento de Prehistoria e Historia Antigua, UNED, 28040 Madrid, Spain

|| Laboratoire de Paléobotanique, UA 327, CNRS, USTL, 3400 Montpellier, France

AMONG things that most strikingly distinguish modern humans from other hominids and the rest of the animal kingdom is the ability to represent things and events pictorially. Complex paintings of the type discovered in the Altamira, El Castillo, Niaux and Lascaux caves represent an important stepping stone in the cultural evolution of humankind. Until now dates were derived from style or dated remains left by prehistoric visitors and could be biased by prolonged occupation or visits unrelated to painting activity. Here we report the first radiocarbon dates for the charcoal used to draw stylistically similar bison in these caves: $14,000 \pm 400$ yr BP in the Spanish caves of Altamira, $12,990 \pm 200$ yr BP in El Castillo, and $12,890 \pm 160$ yr BP for a bison of different style in the French Pyrenean cave of Niaux. Our results demonstrate the imprecise nature of stylistic dating and show that painting dates derived from remains of human activities should be used with caution.

Soon after the antiquity of European cave paintings was recognized, examination of the animals depicted suggested that most were painted during the last ice age. Elaborate efforts were

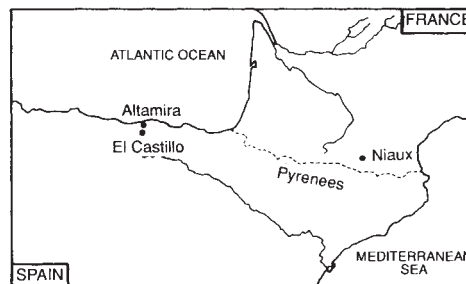


FIG. 1 Location of the Altamira, El Castillo and Niaux caves.

made to develop a floating chronology based on stylistic criteria^{1,2}, an approach that produced conflicting chronologies and raised several fundamental questions about the assumptions underlying art-historical dating^{3,4}. Chronologies based on the nature of fauna represented² have foundered, partly for lack of correlation between the animals shown and animals hunted⁵.

During the past 40 years some progress was made in anchoring the floating chronologies to radiocarbon dates from human occupation remains found near the paintings, but the results lacked precision. Unlike flint implements and portable decorated objects which can sometimes be dated or are frequently found close to dateable remains, only exceptionally can parietal art be linked to dated extraneous material. Perusal of our own and published dates^{1,2,6-9} (Table 1) shows that the debris left by cave visitors or dwellers stretches over many millennia. So the original question remained: when were the paintings produced during these long time intervals?

Consequently, the need to date the pigment itself became apparent. The three most common pigments are red Fe_2O_3 and black MnO_2 or charcoal. To date such charcoal by the traditional ^{14}C technique of β -activity measurements is impractical as the sample requirements would damage the artwork. Recently use has been made of accelerator mass spectrometers, which separate and count individual carbon isotopes and require much less material, to circumvent this problem^{3,10-12}. We have tested this technique on pigment from the French cave of Cougnac and found the drawing to be ~14,300 years old, somewhat younger than previously believed³.

We are reporting the first dates for the charcoal used in

TABLE 1 Description of dated remains of human occupation

	Date (yr BP)
Altamira	
Bone fragment (M829) ⁶	15,500 ± 700
Bone fragment, Magdalenian level (GifA 90047)	14,520 ± 260
Engraved bone fragment, same level (GifA 90057)	14,480 ± 250
Bone fragment, Solutrean level (GifA 90045)	18,540 ± 320
El Castillo	
Perforated engraved bone baton (OxA 970) ⁷	10,310 ± 120
'Cantabrian' bone harpoon (OxA 972) ⁷	12,390 ± 130
Ornamented bone spearhead (OxA 971) ⁷	16,850 ± 220
Réseau Clastres (Niaux)	
Surface finds in the vicinity of wall paintings	
1st charcoal fragment (GifA 89167)	4,760 ± 110
2nd charcoal fragment (GifA 89141)	5,030 ± 110
3rd charcoal fragment (GifA 89157)	7,430 ± 130
4th charcoal fragment (GifA 89143)	9,500 ± 110
5th charcoal fragment (Gif 1938) ⁸	5,650 ± 200
6th charcoal fragment (Gif 1937) ⁸	9,850 ± 230
7th charcoal fragment (Gif 1939) ⁸	10,100 ± 250
8th charcoal fragment (Gif 1940) ⁸	10,150 ± 200
9th charcoal fragment (Ly 621) ⁹	4,590 ± 280

paintings of the Altamira, El Castillo and Niaux caves (Fig. 1), which together with Lascaux contain the finest examples of Franco-Cantabrian 'cave art'¹. They continue to be the subject of a voluminous literature, some of it dealing with the pigments and painting techniques¹³⁻¹⁷. To obtain the amount of carbon needed for dating, we scraped one or several dark sections with a nickel-plated scalpel until a bulk corresponding to ~20–40 mg was collected. In most instances the total sampled area was between 10 and 50 mm². To free the scrapings of calcite contamination we placed each sample in an ultrasonic bath containing 0.2 N HCl; material left undissolved after ~1 hour was collected on a pre-cleaned quartz-fibre filter. The thin black residue coating the filter was then washed with a weakly alkaline solution (to get the 'humic acid' fraction). The filter and the remaining residue were next washed with 0.2 N HCl to remove any CO₂ that might have been absorbed during the alkaline wash, then with water. The washing was followed by heating under a controlled oxygen flow during one hour at 300 °C to remove the modern organic pollutants. Subsequent treatment followed established procedures¹⁰. For more details, our Cougnac paper may be consulted³. Optical microscopy, used to confirm the presence of charcoal in our samples, revealed that, as in the case of Niaux¹⁷, the Cantabrian charcoal came from a coniferous

TABLE 2 Description and ages of pigment samples

	Gross sample weight (mg)	Dateable carbon (mg)	Date (yr BP)
Altamira			
Centre of painted ceiling (zone III in fig. 133 or fig. 670)*			
Small bison facing left:	25.1		
Charcoal (GifA 91178)		0.87	13,570 ± 190
'Humic acid' fraction (GifA 91249)		0.66	14,410 ± 200
Large bison facing left:	21.0		
Charcoal (GifA 91179)		0.70	13,940 ± 170
'Humic acid' fraction (GifA 91254)		1.38	14,710 ± 200
Large bison facing right:	21.2		
Charcoal (GifA 91181)		1.49	14,330 ± 190
'Humic acid' fraction (GifA 91330)		0.53	14,250 ± 180
El Castillo			
Wall near the entrance (zone IV, Nos. 18 and 19 in fig. 139)*			
Large bison facing right†:	5.7		
Charcoal (GifA 91004)		0.82	13,060 ± 200
Large bison facing right‡:	10.6		
Charcoal (GifA 91172)		0.69	12,910 ± 180
Niaux			
The 'salon noir' (panel I in fig. 154)*			
Large bison facing right§:	20.9		
Charcoal (GifA 91319)		0.51	12,890 ± 160
'Humic acid' fraction (GifA 91173)		0.58	12,440 ± 190

* All figure numbers refer to maps, drawings and photographs in ref. 1.

† This had a left-hand outline superimposed on the neck and overlapped two horses.

‡ This showed four human hand imprints on its back.

§ This had two spear marks on its right flank (Fig. 592 in ref. 1).

tree (probably juniper or pine). The choice of panels to be sampled was aided by studies^{14,15} identifying areas where charcoal was detected. A similar study made us realize that dating Lascaux paintings will be difficult owing to the sparse use of charcoal¹³. Our pigment dates are presented in Table 2.

At the Spanish Cantabrian cave of Altamira we sampled three bisons at the centre of the painted ceiling. The high water, clay and limestone content of the scrapings made the separation and purification of the charcoal difficult. This may in part explain the range of dates from ~13,600 to ~14,300 yr BP, with a mean overall of 14,000 ± 400 yr BP, for stylistically similar drawings. To test for contamination with modern organic matter we dated separately the so-called humic acid alkali-soluble fractions¹⁸ and obtained a mean of 14,450 ± 200 yr BP. Both dates are consistent with the attribution of these bisons to Early Style IV. Comparison with the dates shown in Table 1 for bones from the Solutrean and Cantabrian Early Magdalenian¹⁹ strata excavated at the cave entrance indicates that the bisons were the work of Magdalenian occupants.

At the Cantabrian cave of El Castillo two adjacent bisons were dated to 13,060 ± 200 and 12,910 ± 180 yr BP respectively. Here the evidence is strong that the two were painted during the Cantabrian Early Magdalenian period, possibly by the same artist or team. Data in Tables 1 and 2 show that the bisons were painted within a timespan encompassed by dates of portable art objects⁷ by people closer in time to those who made the bone harpoon than by occupants responsible for the ornamented spearhead. Although the sampled El Castillo bisons are said to show stylistic affinities with those dated at Altamira¹, it would



FIG. 2 Picture of the bison sampled at Niaux (N. Aujoulat, Centre National de Préhistoire, Périgueux).

seem that the latter were painted by individuals living many generations earlier.

At the French Pyrenean cave of Niaux, a bison painting (Fig. 2) in the 'Salon noir' was found to be $12,890 \pm 160$ years old. The associated humic acid fraction gave a date of $12,440 \pm 190$ yr BP. These values are close to dates proposed for similarly decorated bone objects, assigned to the Late Magdalenian¹⁵, from the nearby cave of la Vache in a stratum dated to between 12,540 and 12,850 yr BP²⁰. Our dates leave open the possibility that, contrary to current stylistic chronology, people of the same cultural background were responsible for the El Castillo and Niaux paintings. The dates in Table 1 for the charcoal collected on the ground near similar, distinctly Magdalenian, drawings at the Réseau Clastres, a cave adjacent to Niaux, show how unreliable are painting ages derived from what, in the absence of better evidence, have been taken to be contemporaneous deposits found nearby.

We have shown that even in caves that contain irreplaceable artistic treasures, where the integrity of the frequently thin and fragile drawings is of major concern, radiocarbon dating by accelerator mass spectrometry can be achieved without prejudice to the paintings. We have also pointed out the hazards of dating cave paintings by extrapolation from presumed contemporary human debris. Stylistic dating will remain important in the dating of parietal art for a long time, because most paintings contain none or too little dateable pigment, and other art forms, such as stone engravings and carvings are unlikely to be dateable in the foreseeable future. By examining the same animal in three different caves we have made an attempt to test the reliability

of current stylistic chronologies^{1,2,21-23}. As the number of dated paintings increases, revised sets of stylistic criteria should emerge. □

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Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs

Hiroyuki Meguro^{*†}, Hisashi Mori^{*}, Kazuaki Araki^{*}, Etsuko Kushiya^{*}, Tatsuya Kutsuwada^{*†}, Makoto Yamazaki^{*}, Toshiro Kumanishi[‡], Masaaki Arakawa[†], Kenji Sakimura^{*} & Masayoshi Mishina^{*§}

Departments of ^{*}Neuropharmacology and [‡]Neuropathology, Brain Research Institute and [†]Department of Medicine II, School of Medicine, Niigata University, Asahimachi 1, Niigata 951, Japan

THE glutamate receptor (GluR) channel plays a key part in brain function^{1,2}. Among GluR channel subtypes, the NMDA (N-methyl-D-aspartate) receptor channel which is highly permeable to Ca²⁺ is essential for the synaptic plasticity underlying memory, learning and development^{3,4}. Furthermore, abnormal activation of the NMDA receptor channel may trigger the neuronal cell death observed in various brain disorders^{5,6}. A complementary DNA encoding a subunit of the rodent NMDA receptor channel (NMDAR1 or $\zeta 1$) has been cloned and its functional properties investigated^{7,8}. Here we report the identification and primary structure of a novel mouse NMDA receptor channel subunit, designated as $\epsilon 1$, after cloning and sequencing the cDNA. The $\epsilon 1$ subunit shows 11-18% amino-acid sequence identity with rodent GluR channel subunits that have been characterized so far and has structural features common to neurotransmitter-gated ion channels. Expression from cloned cDNAs of the $\epsilon 1$ subunit together with the $\zeta 1$ subunit in *Xenopus* oocytes yields functional GluR channels with high activity and characteristics of the NMDA

receptor channel. Furthermore, the heteromeric NMDA receptor channel can be activated by glycine alone.

Several cDNAs encoding a putative GluR channel subunit have been isolated by series of screening of mouse forebrain cDNA libraries using mouse GluR $\alpha 1$ (GluR1) and $\alpha 2$ (GluR2) subunit cDNAs and newly isolated putative GluR channel subunit cDNA as probes⁹⁻¹². Figure 1 shows the deduced amino-acid sequence of one of these subunits, the $\epsilon 1$ subunit, compared with those of the NMDAR1 subunit of the rat NMDA receptor channel⁷, the $\alpha 1$ subunit of the mouse α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective GluR channel⁹ and the $\gamma 2$ subunit of the mouse kainate-selective GluR channel¹¹. The initiator methionine is assigned to the first methionine residue found in a large open reading frame. The amino-terminal hydrophobic segment is assumed to represent a signal peptide. The proposed mature $\epsilon 1$ subunit is composed of 1,445 amino acids with a calculated relative molecular mass (M_r) of 163,267. Rodent GluR channel subunits thus far characterized⁷⁻¹⁷ can be classified into several subfamilies according to the amino-acid sequence homology (Table 1). Members of the α subfamily form homomeric or heteromeric channels selective for AMPA^{9,13,14}. The β and γ subfamilies include the subunits of the kainate-selective channel^{10,11,16,17}, whereas the ζ subfamily represents the NMDA receptor channel^{7,8}. The function of the δ subfamily is not known. The mouse $\epsilon 1$ subunit shares 11-18% amino-acid sequence identity with these GluR channel subunits (Table 1). The low sequence homology of the $\epsilon 1$ subunit indicates the presence of a novel subfamily of the GluR channel. The $\epsilon 1$ subunit is notably larger in the carboxyl-terminal region.

Analysis and comparison of the local hydropathicity profile of the $\epsilon 1$ subunit suggest the presence of four putative transmembrane segments (M1-M4) in the middle of the molecule (Fig. 1). In agreement with this model is the finding that Arg 586 in the M2 segment of the $\alpha 2$ subunit determines the Ca²⁺ permeability of the AMPA-selective GluR channel^{18,19}. The corresponding position in the $\epsilon 1$ subunit is occupied by an asparagine (residue 595), as is also the case for the $\zeta 1$ (NMDAR1) subunit

§ To whom correspondence should be addressed.

of the NMDA receptor channel^{7,8}. The $\epsilon 1$ subunit contains a lysine at residue 584 in segment M2. The presence of this lysine is confirmed in six independent clones. This positively charged residue in a putative channel-forming segment could influence the ion permeation process. The carboxy-terminal region of

segment M3 is highly conserved among GluR subunits and so may represent an important functional domain. In addition to the putative transmembrane regions, the region preceding segment M1 is also well conserved, and has been proposed to contain the agonist binding site^{9,20}. A point mutation introduced

FIG. 1. Deduced amino-acid sequence of the mouse $\varepsilon 1$ subunit and alignment with those of the mouse $\alpha 1$ (ref. 9), mouse $\gamma 2$ (ref. 11) and rat NMDAR1 (ref. 7) subunits of the GluR channel. Amino-acid residues are numbered beginning with the amino-terminal residue of the mature subunit and the preceding residues are indicated by negative numbers. Numbers of the amino-acid residues at the right-hand end of the individual lines are given. Sets of identical amino-acid residues among four subunits are enclosed and those between the $\varepsilon 1$ and NMDAR1 subunits are shown by vertical bars. The putative trans-membrane segments (M1–M4) are indicated. Potential phosphorylation sites for Ca^{2+} -calmodulin-dependent protein kinase type II are marked with open circles. The point mutations $\alpha 1$ -K445E (ref. 19) and $\alpha 2$ -R586Q (refs 18, 19, 32) affecting the agonist binding and channel properties, respectively, are indicated by arrowheads. Nucleotide sequence data will appear in the DDBJ, EMBL and GeneBank Nucleotide Sequence Database under the accession number D10217.

METHODS. Series of screening of mouse forebrain cDNA libraries in λ gt10 under low-stringency conditions with mouse GluR channel subunit cDNAs (refs 9–12) as probes yielded several cDNA clones encoding a novel GluR channel subunit. The cDNA insert of one of these recombinant phage carrying the $\epsilon 1$ subunit cDNA, λ A19, was subcloned into the *Eco*RI site of the plasmid pBluescript II SK(-) (Stratagene) to yield the plasmid pGRA19. Additional $\epsilon 1$ subunit cDNA clones were isolated by screening under high-stringency conditions using A19 and AT11 (see below) cDNAs as probes. The cDNA inserts of seven recombinant phage were subcloned into the *Eco*RI site of the plasmid pBKSA (ref. 8) to yield the plasmids pAT4, pAT11, pAT12, pAT19, pAT20, pAT201 and pAT202. The complete nucleotide sequences of the cDNA clones A19 (nucleotide residues -327 to 3,181) and AT19 (2,859 to 5,470) were determined by the dideoxy chain termination method³³ using nested deletions and synthetic primers; nucleotide residues are numbered in the 5' to 3' direction, beginning with the codon specifying the amino-terminal residue of the mature $\epsilon 1$ subunit, and the preceding residues are indicated by negative numbers. Partial nucleotide sequences of the clones AT4 (residues 2,550 to 3,432), AT11 (2,550 to 3,622), AT12 (2,404 to 3,622), AT20 (2,550 to 3,177), AT201 (3,526 to 4,401) and AT202 (3,409 to 4,371) are in complete agreement with those of the clones A19 and AT19. Nucleotide and amino-acid sequences were analysed using GENETYX software (SDC) unless otherwise specified.

		-11	
a1	MPYIAFAAFCCTGFLGAYGCA-----HFPNNIGICGLFPNQSSQENAAAFALSO--LTPPK-LLPQIDIV-KISDS		49
r2	MPAAELLLLIVAFANPSCOVLSLSRRAALDDOTVCGRGELALALAREOINGIIE--VPAAK-VEVDIFV-GRDSO		57
c1	NGRLGVYLLVLPALPLVYVNGPAQA-AAEKGPALNIAVLLGNHSDVTERELRNLQPEQATGLPQVYVYALLNRRTPD		60
HMDAR1	HSTHMLTLFALLPSCSFAR-----AACDPKIVNIGAVSLTRKNEQNFREAVNQ-ANKRNGSVKIQLNATSVT-NKPNA		53
a1	FENTYRFFSQFSKGVYVAF-----GFYERTYVHMLTSFCGLNVCFTIPSPFYDTSNQ---FV-LQLRPELOE---	113	
r2	YETTDTHQDILPKGVSVL-----GPSSSPASASTVSNICEKEIPNIVKGPETPRLOYLRASVSLYPSHEDVSL	129	
c1	KSLITHYCDLHSGARINGLYFGD--DTQEAVAOHLDFISSQFTIPILGINGCASHMADKPTSTFFQFGASIQQA-T	137	
HMDAR1	IOMALSVLEDLISSQVYAILVSNPPTPDHFTPTPVSYTAGFYRIPVLCITTRMS-IYSDKSIHLSLRTVPVPSNQS-S	131	
a1	ALISIDHNYKQTFVYIYDADRGLSVLQRYLDT-AAEK--NVQVTAVNLT7TEEGYRMLFODLEKKERLVYVDCESER	190	
r2	AVSRILKSFHNPASLICKAAECLLRLEELVRG-FLIS--K-ETLSYRMLDSDRPTPLL-KEIRDDXVSTIIDANASI	204	
c1	VHLKIHQDYDHHVSLVTTIPFGYRDFISFKITTYDHSFYGVDMQRYITLQTSFEDAKTQ-VOLKHNSSVILLYCSKDE	216	
HMDAR1	VVFENHRYVHNNIILVSDNNHGRAAQRLETLLER--ESKAENVLOFDPGKNHVTALLHEARLEARVILLSAEDD	209	
a1	LNALGQIVKLEKNGICYBYILANLGFMDIDHFKFESGANVTGFLQVNYTDTIPAKIHQQVTSDDADHTRVDYKRPKY	270	
r2	SNLVLRKASLHTSAFYKVIITMDFFILMLDGIYEDSSNIGLGFHNTSNFFYPEFVRSLNHSVRENCEASTYGPAL	284	
c1	AVLILSEARSLTGYOFFVIVPSLVSGATEL-IPKEFPGLISVSDVDVSLAERVRDGLGITTAASSHLEKFSVIP	295	
HMDAR1	AATVYRAAALHTGTCGYVVLVGEREISGHAL--RYAPDGIQLQLIN-KNESANISDAYGYVAQAVKELLE-ENIT	284	
a1	TSALTVDGVYKHAFAFOSLRRORIDIS-RRGNAGDCLANPAVVPQGGIDIGRA--QOVFE--GLTGNVQFNEKGRTHY	346	
r2	SAALHFDVHVVYSVARELNRSO-EIG-VK--PLAC--TSANIVPHTSLNHYL-RHYEVD--GLTGRVFNHSGQRTNY	355	
c1	EAKASCYQOTEN-PETPLHTLQFHVHVTVDGND-LSFTEEGYQVHPRLVIVLHNDREVEKYKAVE-NOTLRL-RHAVV	371	
HMDAR1	DPRECVGHTIIVKTCGLPKRVLSHSSYADGVYGRVEHGGGRKFANYSINML-QRRLLVQVGLYNGTHVPHDKRIIV	363	
a1	TLHVIENED---GIRKIGYVNEDDKFYPAATDAQ-AGGDNSSVQRTYIVTTIL-EDPYVMLKKNANOFEGNDRYDQV	421	
r2	TLRILEKSRQ---GHREIGVVSRTLANHATTLD-IALSQ-TLAKTLYVTITL-ENPVVHRRPHFOALSGNERFQDQ	429	
c1	PRYKFSFQCE-PDDHNLISVTLLEEAPFYVEDIDPLETETC-----VNTYVCRKFV-----KINNSTEGHMYKCKC-GLC	441	
HMDAR1	PGGETENPRGYQNSTRLKIVTIHQEPFVYVKPMS-DCTCKEEFTVHGDPVKYVICTGPHDTPSGSPRHTVPQCCY-GLC	441	
a1	VELAAEIAKHYGVSRLHIVSDHCKYCARP-----DTAAHETVGLVYGRADVAVAPLTITLYKEEVIDFSNFFHSLGIS	497	
r2	VQMLRELAELLRLHRLRVNGLVGLYCAPE-----NGSVNNGGLINRKAOLAVAAFTITAEKVIDSKPFTLGLGIS	514	
c1	IDILKLSRTVFTYDLYLTHCKNG--KKVNV--VMEHIGEVYGRAPVAVYGLSTIHEERSEYVDFSPFYVETGIS	505	
HMDAR1	IDLLIKLARTHFTVEVNLVAKGFTQERVNNSHKEKMEHEDGLSCGDHIVAPLTINKHAAOYIDFSNFFVGLT	521	
a1	INIKKPKQSKPGVFSPLDPLAYELICI-VFAYIGVSVLVFLVSRFSPEYEVNSEEFEEGRDQTSDOOSEHFGIFNSLFS	576	
r2	ILVYVHNRKRPYGSFLNLPSPAVLFLN-LLAYLAVSCVLFLAARLSPEYVYVNH--PCLARPHILENOYTLGSLNFP	581	
c1	VHYSR-SRGTVSPSARLQFSASVYHNFVHLLIYSAIVFVFEYFSPVGY-----NRRLAKGAPHGPFSTIGKAILLL	589	
HMDAR1	ILVKN-EIPRSTLSEHGFQSTLGLV-GLSVNVVAVHLYLDRFSF-----GRFKVNSEEEEDALTSSASFS	592	
		M1	M2
a1	LAFFHQG-CDISRLSGRIVGVVVFTHIISSTYANLAALTVRMYIESAD--LAKQTE---IAYGILEAG	649	
r2	VQGFHQG-SEVFRALSTRCVSYVVAFTLHIISSYANLAALTVQRNEVPYESAD--LADQTH---IEVGTINAG	654	
c1	VGLVFNHSPYVQDQCTSKINVSVAFFAVFLASSTYANLAALHIEEFYQDQVGLSDKFKORPNDSYPPFRFGQVPG	669	
HMDAR1	VEVLLNSGICGARSFSARILGKVAAGHIVASTYANLAALVLDREPERITGIMPRLRAP--KPIYATKQS	669	
		M3	
a1	STKEFFRSEKIAVFENIVTYNKSAPSVFYRTTEGNIIRYKSKNHYLLESTHNEVI--EQRKPDQTHVY-G-HLDS	725	
r2	STHTFFQHSRYQVQVRYHMYGSKSVFYVKSTEEGIAVRLNS--RYAFLLESTHNEY--NRRLNLQTOIE-G-LLOT	727	
c1	STERNIRN--E-VPYH--QVSTKFO-RGVEDALVSLKTH-LDAFIVDAVLYN-KAGROEGGLVLTISGVIFAT	739	
HMDAR1	SVBIVFR-RQE-LSTGY--RHEKHHY-ESAAEAIQAVRDNK-LHDFIVDSAVLEF-EAS--QKDLVTTG-E-LFFR	737	
a1	KVYCATPKCSALRGPVNDAVHLSEQGYLDKSKSPVYKDEGSGKSGSKDNTSASLSRYAVYVILIGGLGLAMLV	805	
r2	KVYCGHPLGSPFDEITAILQLOENHRLKRNHY-EGGR-PREEDNRK--GLNHEGICGVYVILIGLIIVFV	803	
c1	TEGALQKSGVKKRQIDALLQFVGDGENEDLETULT--GIMHEKHEVMS--SOLDIDIMAGVYMLAAAHALSIT	815	
HMDAR1	SGFGCHRGKSPVKNVSLILKSHENGFEEDKTVRY-GEQ--SRSNK--ATITFEHAGVHVLVAGGIVAGIFL	812	
		M4	
a1	ALIEFCYKRSSEHMKGFLIPQOSINEAINTSTPLRNSGAGASGSGSGENGCRVYSODPKSMQIPCMHNSGHPLG	885	
r2	AVNEFIVSTRRSASEEVSVCENHQLRNAVSKRTSRRRRRPGGSRALLSLRAVREHRLSNGLYSAGAGGDAGA	883	
c1	FIVHLYFVYLRFCTFGVCSDRPGLLSISRGIVSYCHGVHIEEEKSPDFHLCGSSNHLKLLRSANNISNHSNHSR	895	
HMDAR1	IFIEIAYKRNKDARRKQNLAFAAVNVYKKNLDRKSGRAEPPDKKATFRAITSTLASSFNRRRSSKOTSTGCGRGALQ	892	
a1	ATGL		889
r2	HGGPORAALDDPGPGGPRPOAPTCTHVRVCOECRRIQALRASGAGAPPRGLGTPAEATSPPRPRPGTGPRELTEH	961	
c1	HDSPKRAADFQRLSDIVHSDYDGNLIVSDNRSPQGDISIFGENHNELOTFYANRKNDSLSHYVFGGNPLTLNESHPH	975	
HMDAR1	NOKDTPLPRAAIEEGQLCSNRHS		920
c1	TVEVAVSTESKGRPRQLVKNHSHESLRLNMQVPYSORDENAEHRTSLKSPRYLPEEYVANSISETSSRATCHREP	1055	
c1	DNHNNHNTDNFRKSHASKYKDCSEVERTVYNTKASSPRDIYIDGKEPFSHLDDPPQFIEHVLPEVDFPDQYQDN	1135	
c1	NEFRKCDSTLPNNRNLNNECDLPNDQVLYAKHFTLKDNGSPNSEGDRYQVSTHCRSCLSNLPTSYGNFTHRSPP	1215	
c1	KCDACLRLHGLYDIDEDQNLQETGNPATREAYQQQVSNHNLQFQKHKLIKINRQSYDMLDRPREDLSPRSISLSK	1295	
c1	DRERLLEGLHLYGSLFVSPSSHLKGNKSLFPOGLEDNRKSHLLPDNTSDNPLFTVGDQRLVIGRCQSDPYKNSLSPQ	1375	
c1	AVYSDLSLSSLSATCSYSDRSGHSDVISENVPIYAANKHNSVTPVLSNCSNRVYKMPSEV	1445	

TABLE 1 Per cent amino-acid sequence identity between GluR channel subunits

α Subfamily				β Subfamily		γ Subfamily		δ Subfamily	ϵ Subfamily	ζ Subfamily
Mouse $\alpha 1$ (GluR1)	Mouse $\alpha 2$ (GluR2)	Rat GluRC (GluR3)	Rat GluRD (GluR4)	Rat GluR5-1	Mouse $\beta 2$ (GluR6)	Rat KA-1	Mouse $\gamma 2$	Mouse $\delta 1$	Mouse $\epsilon 1$	Mouse $\zeta 1$ (NMDAR1)
Mouse $\alpha 1$	66	64	66	36	37	33	32	25	14	18
Mouse $\alpha 2$		72	70	36	37	31	30	23	13	20
Rat GluRC			71	36	38	32	31	22	12	19
Rat GluRD				36	36	31	30	23	13	20
Rat GluR5-1					74	38	38	23	14	21
Mouse $\beta 2$						38	37	21	11	20
Rat KA-1							67	24	14	20
Mouse $\gamma 2$								24	14	20
Mouse $\delta 1$									14	19
Mouse $\epsilon 1$										18

Sequence data are taken from ref. 9 (mouse $\alpha 1$ and $\alpha 2$), ref. 14 (rat GluRC and GluRD), ref. 15 (rat GluR5-1), ref. 10 (mouse $\beta 2$), ref. 17 (rat KA-1), ref. 11 (mouse $\gamma 2$), ref. 12 (mouse $\delta 1$) and ref. 8 (mouse $\zeta 1$). Amino-acid sequence identity between GluR channel subunits was calculated using GeneWorks software (IntelliGenetics). Essentially the same values (differences are within 2%) are obtained for the rat counterparts of the mouse $\alpha 1$, $\alpha 2$, $\beta 2$ and $\zeta 1$ subunits, that is, the GluR1, GluR2, GluR6 and NMDAR1 subunits.

into this region of the $\alpha 1$ subunit of the AMPA-selective GluR channel (mutation $\alpha 1$ -K445E) strongly reduces the EC_{50} (50% effective concentration) value for L-glutamate¹⁹. The putative cytoplasmic domain between segments M3 and M4 contains a potential phosphorylation site for Ca^{2+} -calmodulin-dependent protein kinase type II (ref. 21).

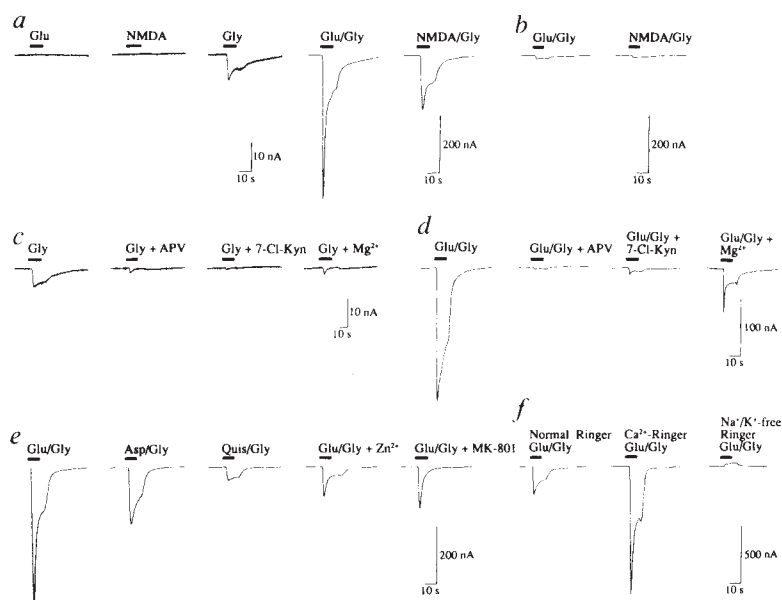
The $\epsilon 1$ subunit-specific messenger RNA was synthesized *in vitro* from cloned cDNA and was injected into *Xenopus* oocytes. The injected oocytes gave no detectable response to 10 μ M L-glutamate and 100 μ M NMDA in the presence of 10 μ M glycine, required for activation of the NMDA receptor channel^{22,23}. But responses were large when the $\epsilon 1$ subunit-specific mRNA was injected together with the $\zeta 1$ subunit-specific mRNA (Fig. 2a). The peak inward currents obtained in normal frog Ringer's solution at -70 mV membrane potential were 364 ± 62 nA (mean $\pm$ s.e.m., $n = 7$) in response to 10 μ M L-glutamate plus 10 μ M glycine, and 175 ± 30 nA ($n = 7$) in response to 100 μ M NMDA plus 10 μ M glycine. The current amplitudes were much larger than those observed for the oocytes injected

with the $\zeta 1$ subunit-specific mRNA alone (8 ± 2 nA, $n = 7$, for 10 μ M L-glutamate plus 10 μ M glycine, and 6 ± 1 nA, $n = 7$, for 100 μ M NMDA plus 10 μ M glycine) (Fig. 2b). The initial peak currents may include Cl^- currents activated secondarily by Ca^{2+} influx, because steady currents were obtained when Ca^{2+} in normal Ringer's solution was replaced by Ba^{2+} . In Ba^{2+} -Ringer's solution, current amplitudes obtained for 10 μ M L-glutamate plus 10 μ M glycine and 100 μ M NMDA plus 10 μ M glycine were 84 ± 4 nA and 57 ± 3 nA ($n = 7$), respectively, for oocytes implanted with the $\epsilon 1$ and $\zeta 1$ subunits, and 2 ± 0.4 nA and 1 ± 0.2 nA ($n = 7$), respectively, for oocytes implanted with the $\zeta 1$ subunit alone. No response was detected for either 100 μ M kainate or 100 μ M AMPA. We conclude that the $\epsilon 1$ protein is a subunit of the NMDA receptor channel.

The functional properties of the heteromeric $\epsilon 1/\zeta 1$ channel were examined using the oocyte expression system. In the absence of glycine, 10 μ M L-glutamate or 100 μ M NMDA elicited little response, whereas 10 μ M glycine alone evoked small but clear current responses (Fig. 2a). The response to

FIG. 2 Functional properties of NMDA receptor channels expressed in *Xenopus* oocytes. The concentrations of agonists and antagonists are as follows: Glu, 10 μ M L-glutamate; NMDA, 100 μ M; Gly, 10 μ M glycine; Asp, 100 μ M L-aspartate; Quis, 100 μ M quisqualate; APV, 100 μ M; 7-Cl-Kyn, 30 μ M 7-chloro-kynurenate; Mg^{2+} , 100 μ M $MgCl_2$; Zn^{2+} , 100 μ M $ZnCl_2$; MK-801, 1 μ M (+)-MK-801. Membrane potential was -70 mV. Inward current is downward. The duration of bath application of agonists and antagonists is indicated by bars without taking into account the dead-space time in the perfusion system (~ 2 s). a, Response of heteromeric $\epsilon 1/\zeta 1$ channels to Glu, NMDA, Gly, Glu plus Gly and NMDA plus Gly. b, Responses of homomeric $\zeta 1$ channels to Glu plus Gly and NMDA plus Gly. c, Effects of APV, 7-Cl-Kyn and Mg^{2+} on the response to Gly of $\epsilon 1/\zeta 1$ channels. d, Effects of APV, 7-Cl-Kyn and Mg^{2+} on the response to Glu plus Gly of $\epsilon 1/\zeta 1$ channels. e, Responses of $\epsilon 1/\zeta 1$ channels to Glu plus Gly, Asp plus Gly and Quis plus Gly, and effects of Zn^{2+} and MK-801 on the response to Glu plus Gly. f, Responses to Glu plus Gly of $\epsilon 1/\zeta 1$ channels recorded from an oocyte in normal frog Ringer's solution (left), Ca^{2+} -Ringer's solution (middle) and Na^+ - and K^+ -free Ringer's solution (right).

METHODS. The 3.5-kilobase-pair (kb) *EcoRI* fragment from pGRA19 was blunted and inserted into the *SmaI* site of the plasmid pSP64AX (ref. 34) in the same orientation as the SP6 promoter to yield the plasmid pSPA19. The 3.0-kb *SalI*-*HindIII* fragment from pSPA19, the 0.36-kb *HindIII*-*NcoI* fragment from pSPA19 and the 5.4-kb *NcoI*-*SalI* fragment from pAT19 were ligated to yield the plasmid pBKSA $\epsilon 1$. The $\epsilon 1$ subunit-specific mRNA was synthesized *in vitro* with T3 RNA polymerase (BRL) using *NotI*-cleaved pBKSA $\epsilon 1$ as template. Transcription was primed with cap dinucleotide 7mGpppG (0.5 mM). *Xenopus laevis* oocytes were injected with the $\epsilon 1$ subunit-specific or the



$\zeta 1$ subunit-specific mRNA (ref. 8) or in combination; the concentration of the respective mRNA was $0.25 \mu g \mu l^{-1}$ and the average volume injected was ~ 50 nl per oocyte. Whole cell currents were recorded after 2–3 days incubation with a conventional two-micropipette voltage clamp as described^{9,19}.

10 μ M glycine was diminished by 100 μ M D-2-amino-5-phosphonovaleate (APV), a specific competitive antagonist of the NMDA receptor²⁴, 30 μ M 7-chloro-kynurenate, a competitive antagonist for the glycine modulatory site of the NMDA receptor²⁵, and 100 μ M Mg^{2+} , known to block the NMDA receptor channel in a voltage-dependent manner^{26,27} (Fig. 2c). The responses to 10 μ M L-glutamate plus 10 μ M glycine were also attenuated by these inhibitors (Fig. 2d) and by 100 μ M Zn^{2+} , reported to induce a noncompetitive inhibition of the NMDA response in a voltage-independent manner^{28,29} and 1 μ M (+)-MK-801, a channel blocker of the NMDA receptor³⁰ (Fig. 2e). In the presence of glycine, L-aspartate and quisqualate also evoked responses (Fig. 2e). The heteromeric $\epsilon 1/\zeta 1$ channels showed a clear inward current in Na^+ - and K^+ -free Ringer's solution containing 20 mM Ca^{2+} (Ca^{2+} -Ringer's solution¹⁹), whereas a marginal outward current was observed in control Na^+ - and K^+ -free Ringer's solution (Fig. 2f), indicating that the heteromeric channels are permeable to Ca^{2+} . These pharmacological and electrophysiological properties are in good agreement with those of the NMDA-type GluR channel^{1-3,22-30}. One unexpected finding is the action of glycine as an agonist.

The expression patterns in adult mouse brain of the $\epsilon 1$ subunit as well as the $\zeta 1$ and $\zeta 1-2$ subunits⁸ of the NMDA receptor channel were visualized by *in situ* hybridization (Fig. 3a). For the $\epsilon 1$ subunit-specific oligonucleotide probe, distinct signals were observed in pyramidal cells of the hippocampal gyrus and

in granule cells of the dentate gyrus through microscopic examination of the autoradiographed sections. Less distinct signals were also seen in various neurons of other cerebral structures, including the cerebral cortex, the caudate-putamen, the thalamus and the amygdaloid nuclei. In the cerebellar cortex, there were signals in the granule cell layer. The specificity of the signals were verified by control hybridization in the presence of unlabelled probes in excess. Some nonspecific signals were noted, often in acellular areas. Signals obtained with the $\zeta 1$ subunit-specific and the $\zeta 1-2$ subunit-specific oligonucleotide probe distributed widely in various neurons in the cerebrum and the cerebellum, largely overlapping with the distribution of the $\epsilon 1$ subunit mRNA. Blot hybridization analysis of total RNA from mouse forebrain identified RNA species of $\sim 18,000$, $\sim 4,100$ and $\sim 4,000$ nucleotides for the $\epsilon 1$, $\zeta 1$ and $\zeta 1-2$ subunits, respectively (Fig. 3b).

Our investigation has revealed the presence of a novel NMDA receptor channel subunit $\epsilon 1$. The combination of the $\epsilon 1$ subunit and the previously identified $\zeta 1$ subunit yields highly active GluR channels with characteristic of the NMDA receptor channel, such as a requirement of glycine and inhibition or blocking by APV, 7-chloro-kynurenate, Mg^{2+} , Zn^{2+} and (+)-MK-801. Current-voltage relationships determined in normal Ringer's solution and Ba^{2+} -Ringer's solution in the presence and absence of 100 μ M $MgCl_2$ indicate that Mg^{2+} blocking is voltage-dependent. The newly found $\epsilon 1$ subunit is much larger than other

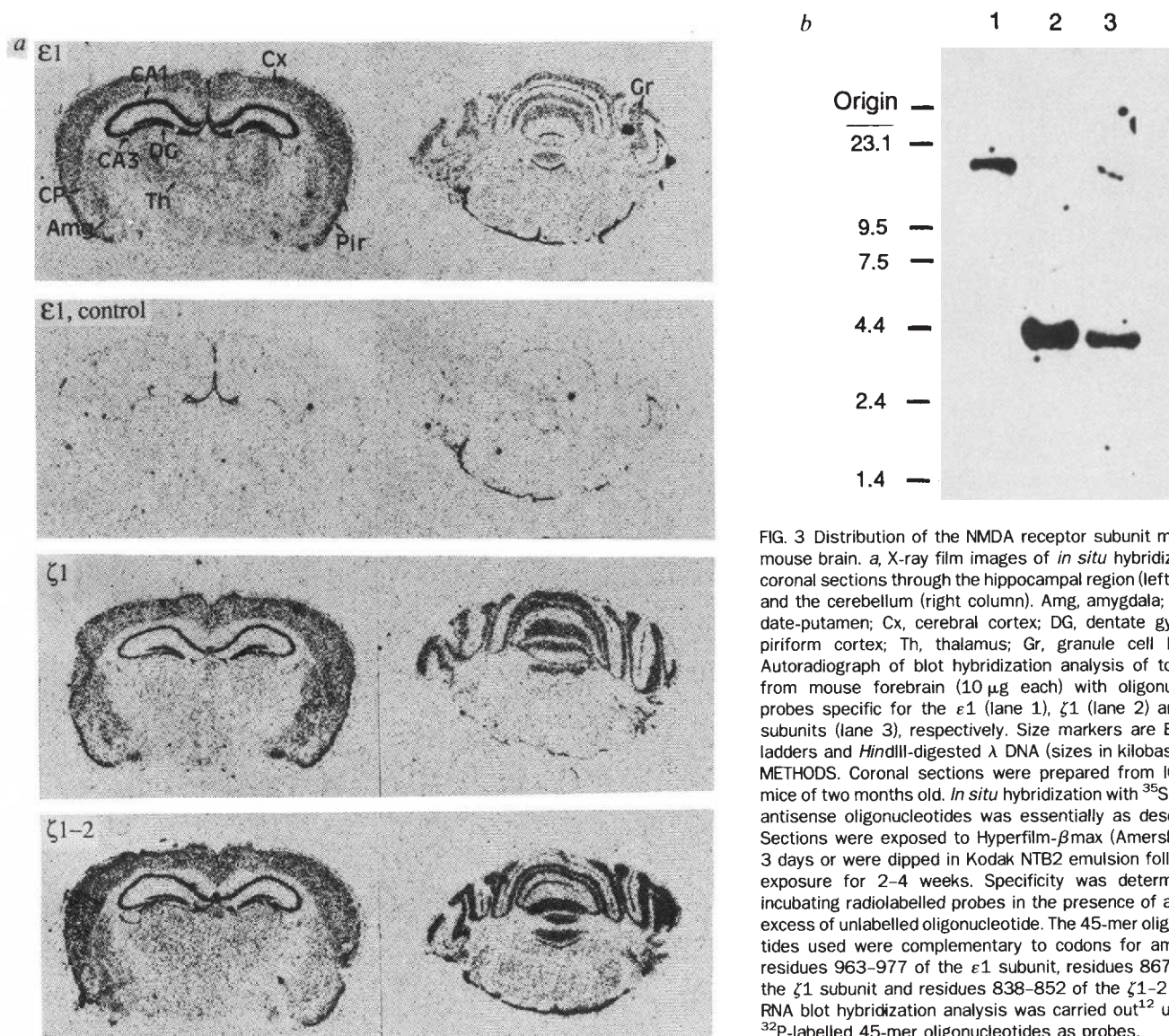


FIG. 3 Distribution of the NMDA receptor subunit mRNAs in mouse brain. *a*, X-ray film images of *in situ* hybridization of coronal sections through the hippocampal region (left column) and the cerebellum (right column). Amg, amygdala; CP, caudate-putamen; Cx, cerebral cortex; DG, dentate gyrus; Pir, piriform cortex; Th, thalamus; Gr, granule cell layer. *b*, Autoradiograph of blot hybridization analysis of total RNA from mouse forebrain (10 μ g each) with oligonucleotide probes specific for the $\epsilon 1$ (lane 1), $\zeta 1$ (lane 2) and $\zeta 1-2$ subunits (lane 3), respectively. Size markers are BRL RNA ladders and *Hind*III-digested λ DNA (sizes in kilobases). METHODS. Coronal sections were prepared from ICR male mice of two months old. *In situ* hybridization with ^{35}S -labelled antisense oligonucleotides was essentially as described³⁵. Sections were exposed to Hyperfilm- β max (Amersham) for 3 days or were dipped in Kodak NTB2 emulsion followed by exposure for 2–4 weeks. Specificity was determined by incubating radiolabelled probes in the presence of a 20-fold excess of unlabelled oligonucleotide. The 45-mer oligonucleotides used were complementary to codons for amino-acid residues 963–977 of the $\epsilon 1$ subunit, residues 867–881 of the $\zeta 1$ subunit and residues 838–852 of the $\zeta 1-2$ subunit. RNA blot hybridization analysis was carried out¹² using the ^{32}P -labelled 45-mer oligonucleotides as probes.

GluR channel subunits identified so far. In this context, it is interesting that molecular target size analysis of the rat NMDA receptor complex has identified a protein of M_r 209,000 (209K) protein for [^3H]-3-($\pm$)-2-(carboxypiperazin-4-yl)propyl-1-phosphonic acid binding, in addition to a 121K protein for [^3H]-L-glutamate binding³¹.

The availability of the $\epsilon 1$ and $\zeta 1$ subunit cDNAs makes it possible to investigate fundamental properties of the NMDA receptor channel. L-Glutamate (10 μM) or NMDA (100 μM) alone evokes little response, in agreement with the notion that glycine is absolutely required for the activation of the NMDA

receptor channel^{22,23}. In contrast, glycine (10 μM or even 1 μM) alone activates the $\epsilon 1/\zeta 1$ heteromeric NMDA receptor channel. Although contamination by L-glutamate or L-aspartate of the glycine solution might explain the response, this possibility is unlikely because dose-response relationships show that the EC_{50} value for glycine is around 2 μM and that the current amplitude evoked by 100 μM or 1 mM glycine is comparable with that obtained by 10 μM glycine. These results clearly indicate that glycine can act as an agonist on the $\epsilon 1/\zeta 1$ NMDA receptor channel. This does not exclude a possible modulatory action of glycine. $\square$

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Calcium-dependent enhancement of calcium current in smooth muscle by calmodulin-dependent protein kinase II

John G. McCarron^{*†}, J. Graham McGeown^{*},
Sheila Reardon[‡], Mitsuo Ikebe[‡], Fredric S. Fay^{*†}
& John V. Walsh Jr[†]

^{*} Biomedical Imaging Group, Program in Molecular Medicine and

[†] Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655, USA

[‡] Department of Physiology, Case Western Reserve University, College of Medicine, Cleveland, Ohio 44106, USA

CALCIUM entry through voltage-activated Ca^{2+} channels is important in regulating many cellular functions. Activation of these channels in many cell types results in feedback regulation of channel activity¹. Mechanisms linking Ca^{2+} channel activity with its downregulation have been described^{2,3}, but little is known of the events responsible for the enhancement of Ca^{2+} current that in many cells follows Ca^{2+} channel activation and an increase in cytoplasmic Ca^{2+} concentration^{4,5}. Here we investigate how this positive feedback is achieved in single smooth muscle cells. We find that in these cells voltage-activated calcium current is persistently but reversibly enhanced after periods of activation. This persistent enhancement of the Ca^{2+} current is mediated by activation of calmodulin-dependent protein kinase II because it is blocked when either the rise in cytoplasmic Ca^{2+} is inhibited or activation of calmodulin-dependent protein kinase II is prevented by specific peptide inhibitors of calcium-calmodulin or calmodulin-dependent protein kinase II itself. This mechanism may be important in different forms of Ca^{2+} current potentiation, such as those that depend on prior Ca^{2+} channel activation or are a result of agonist-induced release of Ca^{2+} from internal stores.

We used enzymatically dispersed single smooth muscle cells from the stomach muscularis of *Bufo marinus* which were suitable for investigating the pathway responsible for enhancing calcium current (I_{Ca}) using methods which we had developed to study Ca^{2+} currents⁶, Ca^{2+} signalling⁷ and Ca^{2+} -dependent protein kinases⁸. In the standard protocol illustrated in Fig. 1a, the inward calcium current evoked by a 3-second depolarizing test potential to 0 mV was increased in peak magnitude and slowed in its time course of decay when preceded by a train of depolarizing pulses. The enhancement was highly reproducible, occurring in 24 out of a series of 27 cells. In these cells, I_{Ca} increased from a peak value of 425 ± 41 pA to 539 ± 51 pA (mean $\pm$ s.e.m.; $P < 0.0001$) and its half-time of decay slowed from 41.4 ± 3.1 ms to 56.9 ± 4.8 ms ($P < 0.0001$). The increase in I_{Ca} could be elicited up to three times in the same cell, although the magnitude of the effect tended to decrease with repetition. Although $[\text{Ca}^{2+}]_i$ increased from 172 ± 16 nM to 743 ± 67 nM during the train of pulses, Ca^{2+} levels had already returned to resting levels by the time that I_{Ca} was enhanced. Thus, mean $[\text{Ca}^{2+}]_i$ was 187 ± 19 nM before the pre-train pulse and 181 ± 16 nM before the post-train pulse. Enhancement of I_{Ca} was observed in cells showing $[\text{Ca}^{2+}]_i$ elevations ranging from 114 to 1,379 nM above baseline.

As both high-voltage-activated, slowly decaying and low-voltage-activated, rapidly decaying calcium currents are found in smooth muscle cells^{9,10}, we examined which current was enhanced by determining current-voltage relationships before and after trains of depolarizing pulses (Fig. 1b). The low-voltage-activated current, which often appears as a 'shoulder' negative to 0 mV (ref. 11), was not augmented, but the current at positive potentials was clearly enhanced. In fact, whereas the current at +10 mV increased from 543 ± 53 pA to 605 ± 45 pA ($n = 8$; $P < 0.05$), the current at -20 mV actually decreased from 78 ± 16 pA to 59 ± 15 pA ($P < 0.005$). As the low-voltage-activated current decays relatively rapidly¹¹, we also measured the current at 70 ms, a time chosen to diminish the contribution of low-voltage-activated current while preserving an adequate level of current to permit accurate measurement. The 70 ms current is augmented by a greater fraction than the peak current (Fig. 1b): in the

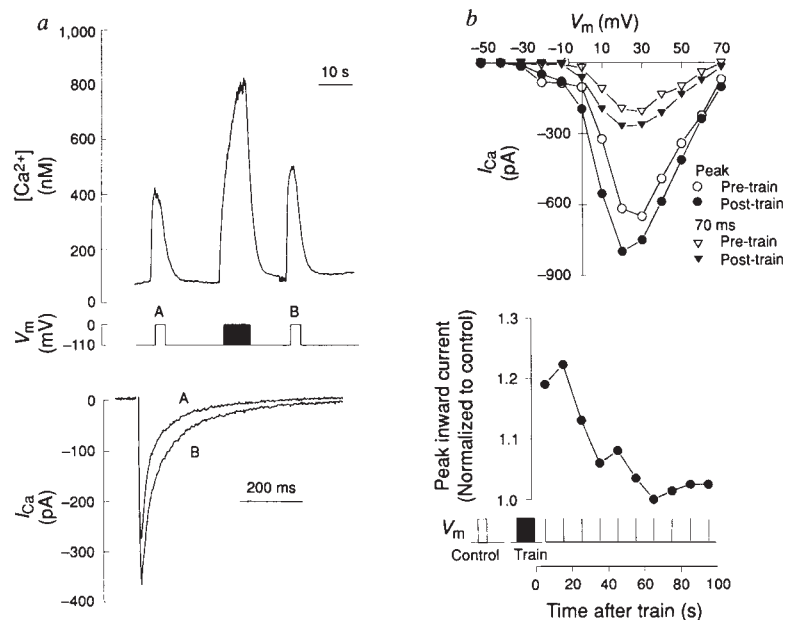
FIG. 1 Effect of repetitive depolarization on subsequently evoked I_{Ca} . **a**, Changes in calcium currents and cytoplasmic Ca^{2+} concentration for a single smooth muscle cell in response to depolarizing command potentials. The lower record shows whole-cell patch-clamp recordings in which the outward K^+ currents have been blocked with caesium chloride in the patch pipette and tetraethylammonium chloride (TEA) in the bath solution. The inward current evoked in response to a 3-s potential step to 0 mV (A on the lower panel) was increased after a train of depolarizing pulses, and its rate of inactivation was slowed (B on the lower panel). Enhancement is observed after Ca^{2+} concentration has returned to pre-stimulus levels (upper panel). **b**, Current-voltage relationships before and after the conditioning protocol for a single cell. To minimize rundown of I_{Ca} , five 100-ms command pulses to different potentials were delivered at a frequency of 0.2 pulses s^{-1} both before and after the conditioning train. This was repeated until the full voltage range had been covered. Plot shows both peak current and isochronic current 70 ms after the onset of the command pulses. **c**, Time course of the enhancement of inward current in a single cell following a train of depolarizing stimuli. A series of command potentials, each only 50 ms in duration to minimize the $[Ca^{2+}]$ rise evoked by each pulse, were delivered at 10-s intervals (beginning 5 s after the train). The resulting peak inward currents have been expressed as ratios of the control value elicited before the train, and plotted against the time after the train.

METHODS. Single smooth muscle cells from the stomach of *Bufo marinus* were prepared by enzymatic dissociation as previously described²⁸. Tight seal, whole cell recordings were made with the following solutions: in the pipette (mM), CsCl 140, MgCl₂ 4, ATP 3.5, Fura 2 free acid 0.1, PIPES (1,4-piperazine-*N,N'*-bis[2-ethane-sulphonic acid]) 20, pH adjusted to 7.15–7.2 with CsOH; (in some experiments the pipette Ca^{2+} concentration was set at 0.1 μ M with a Ca^{2+} buffer (EGTA (ethylene glycol-bis (β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid) 0.25 mM and 0.1 mM CaCl₂), but no differences were observed in calcium currents or calcium transients); in the bath (mM), NaCl 94, KCl 3, CaCl₂ 20, MgCl₂ 1, HEPES (*N*-2-hydroxyethyl-piperazine *N'*-2-ethane sulphonic acid) 10, pH adjusted to 7.4 with NaOH. All experiments were at room temperature. Recordings were made using borosilicate patch electrodes (5–10 Mohm) with an Axoclamp 2A amplifier used in continuous, single-electrode voltage-clamp mode. In the standard protocol, Ca^{2+} currents were measured with respect to the current at the end of the 3-s voltage command pulses. As much as 40% of the series

series of 27 cells tested with the standard protocol shown in Fig. 1a, the 70 ms current was increased from 184 ± 21 pA before the train to 287 ± 29 pA after it ($P < 0.0001$), an increment of $54 \pm 8\%$ compared with $28 \pm 6\%$ for peak current. These results suggest that the enhancement of I_{Ca} reflects an effect on the high-voltage-activated current which is presumably carried in large part by the L-type channels described in these cells¹⁰.

The enhancement of I_{Ca} was reversible (Fig. 1c). In this experiment peak I_{Ca} was maximally enhanced 15 s after the train and returned to the control level after a further 50 s. By contrast, $[Ca^{2+}]_i$ following the train had returned to baseline levels before enhancement of current was maximal. The enhancement of I_{Ca} outlasted the elevation of Ca^{2+} concentration due to the conditioning train of pulses in all 8 cells studied with this paradigm. Hence, either the elevation in $[Ca^{2+}]_i$ is not responsible for the increase in I_{Ca} or otherwise it acts through mediators that have a long persistence.

To distinguish between these possibilities we determined whether the enhancement of the inward current could be elicited under conditions in which a rise in $[Ca^{2+}]_i$ was prevented by either a Ca^{2+} chelator inside the cell or the substitution of another charge carrier for Ca^{2+} ions. Inclusion of the calcium chelator 1,2-bis(*o*-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA) at 10 mM in the patch pipette attenuated the rise in $[Ca^{2+}]_i$ during the conditioning protocol (Fig. 2). No increase in I_{Ca} was observed; peak current averaged 350 ± 74 pA before, and 323 ± 71 pA after, the conditioning protocol ($n = 8$). For the 70 ms current, values were 149 ± 34 pA and 133 ± 26 pA, respectively. Similar decreases were seen in both peak and 70 ms



resistance was compensated. I_{Ca} rose sufficiently slowly so that its peak value was not significantly affected by the capacitive transient, as demonstrated using small (-20 mV) hyperpolarizing pulses with appropriate scaling²⁹. Capacity transients have been blanked for clarity. Data were digitally stored on an IBM AT-type 286 PC (sampling frequency 300 Hz after filtering with a single pole filter, 100 Hz cutoff). Ca^{2+} concentration was measured with a high-time-resolution, digital, dual-wavelength microfluorimeter, recording fluorescence emission at 510 nm and exciting alternately at 340 nm and 380 nm at 105 Hz, as described previously³⁰. Experimental protocols began 5 min after breaking into the cell and, unless otherwise indicated, the interval after the train of pulses was 12 s. The conditioning protocol consisted of a train of depolarizing pulses from -110 mV to 0 mV at a frequency of 2 pulses s^{-1} for 8 s. The statistical significance of differences between means has been tested using a paired *t*-test applied to the absolute values of the measurements made.

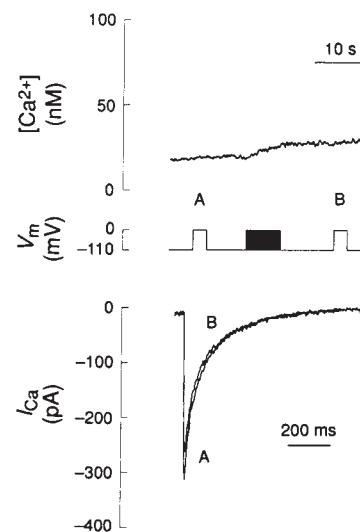
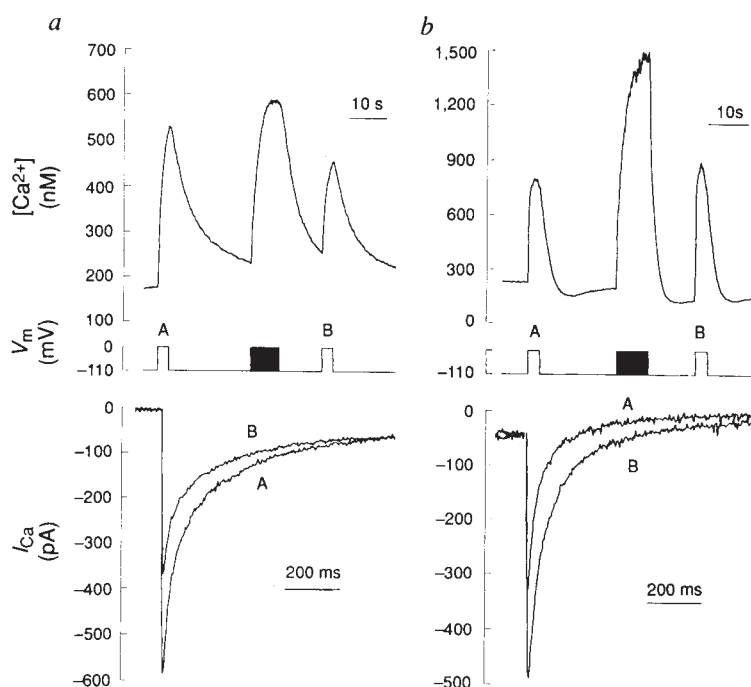


FIG. 2 Effect of a Ca^{2+} chelator on the changes in I_{Ca} and Ca^{2+} concentration in a single smooth muscle cell in response to a 3-s depolarization before and after a train of depolarizing stimuli. The calcium buffer, BAPTA (1,2-bis(*o*-amino phenoxy) ethane *N,N,N',N'*-tetraacetic acid) was included in the standard patch pipette solution at 10 mM. Protocol was identical to that illustrated in Fig. 1a, but the rise in Ca^{2+} concentration during the depolarizing train was greatly reduced. In five such cells Ca^{2+} concentration increased by only 37 ± 20 nM from a resting concentration of 73 ± 34 nM.

FIG. 3 Effect of a calmodulin inhibitory peptide on the enhancement of inward current following a train of depolarizing stimuli. **a**, The peptide calmodulin antagonist RS20, which corresponds to the amino-acid sequence of the calmodulin-binding domain of MLCK (residues 493–512 of smooth muscle MLCK), was included in the pipette at a concentration of 100 μM (sequence Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Ala*-Ile-Gly-Arg-Leu-Ser-Ser; asterisk indicates deleted residue in control peptide used in experiment in **b**) and pressure-injected into the cell from the patch pipette (5–15 psi, 30–100 ms). The resulting increases in cell volume were estimated from digitized video images recorded during the experiment using previously described techniques³¹. These equated to a final RS20 concentration of $49 \pm 8 \mu\text{M}$ inside the cells ($n=7$). This inhibited contraction and completely abolished the enhancement of I_{Ca} . In addition to abolishing the enhancement of I_{Ca} , RS20 markedly reduced the rate of Ca^{2+} removal, as is evident from the upper panel of **a**. Similar effects of RS20 on the rate of Ca^{2+} removal have been reported in these cells after other stimuli; this effect of RS20 presumably reflects inhibition of Ca^{2+} pumps that require calcium-calmodulin for optimal activity³². Note that the RS20 peptide does not simply block the enhancement of I_{Ca} but causes it to decrease. The decrease was not correlated with the level of Ca^{2+} just before pulse B. This diminution in I_{Ca} may reflect the same process which causes a decrease in low-voltage-activated I_{Ca} after the conditioning protocol. **b**, Similar injections of the same concentration (100 μM) of an inactive control peptide (NRS20), almost identical to RS20 but with a single amino-acid deletion (indicated by an asterisk in the RS20 sequence), failed to block the enhancement of I_{Ca} .



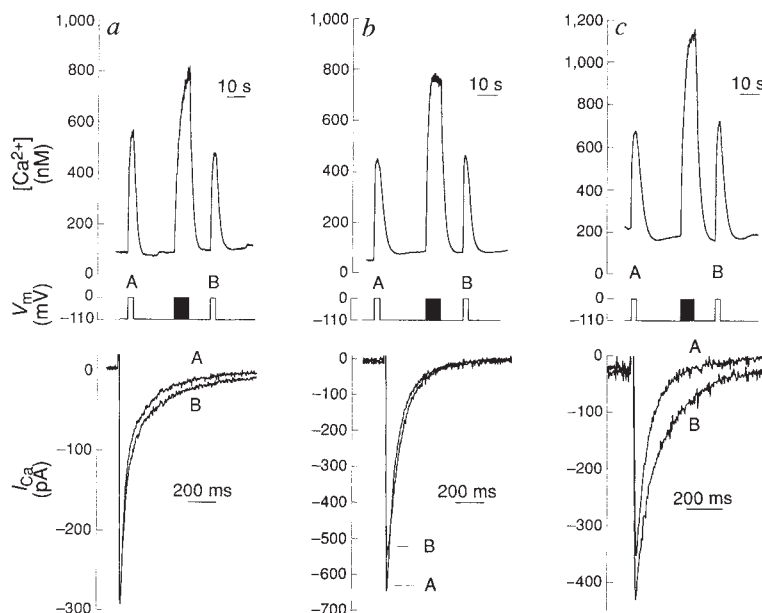
current following the conditioning protocol, when extracellular Ca^{2+} was replaced with Ba^{2+} ($n=3$). Thus, a train of depolarizing pulses is not sufficient to cause an enhancement of I_{Ca} in the absence of an associated rise in Ca^{2+} concentration. Rather, the enhancement of I_{Ca} is a consequence of the elevation of $[\text{Ca}^{2+}]_i$.

Because many of the effects of Ca^{2+} in cells are mediated through its interaction with calmodulin¹² (CaM), we included a synthetic calmodulin inhibitory peptide¹³ (RS20; 100 μM) in the patch pipette solution. RS20 blocked the augmentation of I_{Ca} normally triggered by elevated $[\text{Ca}^{2+}]_i$ (Fig. 3a). The peak I_{Ca} in the presence of RS20 averaged 558 ± 51 pA before the conditioning protocol, and 455 ± 80 pA after it ($P < 0.05$; $n=10$); the 70 ms current was 290 ± 23 pA before and 260 ± 40 pA afterwards (n.s.). A control peptide (NRS20), which has a primary sequence virtually identical to RS20 except for a single amino-acid deletion, but which has only 0.1% of the affinity for

the calcium-calmodulin complex, failed to block the enhancement of I_{Ca} (Fig. 3b). In 11 cells with NRS20, the mean peak I_{Ca} was 218 ± 32 pA and 257 ± 41 pA ($P < 0.04$) before and after the train of depolarizing pulses, respectively, and the 70 ms current was 86 ± 14 and 124 ± 20 pA, respectively ($P < 0.02$). Addition of the organic calmodulin antagonist calmidazolium¹⁴ to the bath (0.5 μM) had effects similar to RS20 in six cells; control experiments using the calmidazolium vehicle, dimethyl sulphoxide (0.05% v/v), showed no effect on the enhancement of I_{Ca} . It seems, then, that interaction of calcium with calmodulin is necessary for the enhancement process.

As the augmentation of I_{Ca} persists after $[\text{Ca}^{2+}]_i$ has returned to control levels (Fig. 1a), and thus after a decline in calcium-calmodulin levels, calcium-calmodulin may trigger a more persistent effect, possibly by activating calmodulin-dependent protein kinase II (CaMPKII)¹⁵. We first established that CaMPKII was present in toad stomach smooth muscle with roughly half

FIG. 4 Effect of two CaMPKII auto-inhibitory domain peptides (CKII and CK3AA) on the induction of persistent enhancement of inward current by a train of depolarizing stimuli. **a**, CKII (corresponds to CaMPKII residues 281–309; sequence: Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala) was included in the patch pipette at 75 μM but the intracellular concentration was probably lower than this as the peptide was allowed to diffuse into the cell. It is to be expected that a considerably higher concentration of peptide would be required to inhibit CaMPKII in the intact cell than under standard *in vitro* conditions because IC_{50} for a pseudo-substrate peptide is likely to be increased by the higher ATP concentration (more than 10-fold higher) expected in the cell³³. **b**, Similar results were obtained when the CaMPKII inhibitor CK3AA (corresponds to CaMPKII residues 277–298 with alanine substituted at positions 279 and 286; sequence: Val-Ala-Ala-Met-Met-His-Arg-Gln-Glu-Ala-Val-Glu-Cys-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys) was pressure-injected into the cell (100 μM in pipette solution). **c**, Effect of inclusion in the patch pipette solution of CKII which was chymotryptically cleaved (equivalent to 75 μM intact CKII). The cleaved peptide lacked CaMPKII blocking effect and did not prevent enhancement of I_{Ca} .



the activity (per gram tissue, wet weight) of that in chicken gizzard¹⁶. As in other smooth muscles, the kinase in toad stomach showed calmodulin-dependent phosphorylation activity against caldesmon. CaMPKII autophosphorylated in the presence of Ca^{2+} and calmodulin. This form had an apparent M_r of 60,000 (60K) and partial calmodulin-independent activity. Most important, we found that two peptides (CKII and CK3AA) corresponding to portions of the regulatory domain of CaMPKII from brain¹⁷, inhibited the isolated enzyme from toad stomach *in vitro* with IC_{50} values of 1.0 and 8.0 μM , respectively. The peptides did not inhibit protein kinases A or C at concentrations up to 300 μM . Whereas CKII had relatively weak calmodulin binding activity (1/20 of that of RS20), CK3AA apparently had none as no inhibitory effect was observed on the calmodulin-dependent enzyme myosin light-chain kinase (MLCK) up to 300 μM .

We therefore used CKII and CK3AA to test whether CaMPKII was involved in the persistent enhancement of I_{Ca} following the conditioning protocol. Inclusion of 75 μM CKII in the patch pipette inhibited the augmentation of I_{Ca} normally produced by increased $[\text{Ca}^{2+}]_i$ (Fig. 4a). In 24 cells tested with this peptide included in the patch pipette, peak current was 260 ± 26 pA before and 258 ± 28 pA after the conditioning protocol, whereas the 70 ms current was 108 ± 15 pA and 124 ± 16 pA (n.s.), respectively. The enhancement of I_{Ca} was also blocked when CK3AA was present in the pipette solution (100 μM ; Fig. 4b) with peak currents of 296 ± 76 pA and 286 ± 79 pA, and 70 ms currents of 113 ± 36 pA and 122 ± 41 pA (n.s.; $n = 10$), before and after the conditioning protocol, respectively. Control experiments used CKII cleaved into two fragments by α -chymotrypsin. The cleaved peptide was inactive against CaMPKII and failed to block the calcium-dependent enhancement of I_{Ca} (Fig. 4c), with peak I_{Ca} increasing from 311 ± 39 pA to 360 ± 53 pA ($P < 0.02$; $n = 13$) and the 70 ms current from 125 ± 15 pA to 173 ± 24 pA ($P < 0.001$; $n = 13$).

These results indicate that the persistent enhancement of I_{Ca} following the conditioning protocol could be mediated by CaMPKII, but we also checked for the possible involvement of other protein kinases. Exposure of cells to 5 mM 8-bromo-cyclic AMP had no effect on I_{Ca} , suggesting that in these smooth muscle cells, unlike adrenal chromaffin¹⁸ or cardiac cells¹⁹, cAMP-dependent protein kinase could not enhance I_{Ca} . We also found that H7, an inhibitor of protein kinase C (10 μM , pre-incubated for at least 15 min), failed to block the enhancement of I_{Ca} induced by the conditioning protocol in 6 of 8 cells tested. Earlier studies¹¹ showed that muscarinic agonists caused an enhancement of high-voltage-activated, slowly inactivating I_{Ca} , an effect mimicked by diacylglycerol analogues and presumably mediated by protein kinase C. The similarity of the effects to those reported here may reflect the similarities in substrate phosphorylation consensus sequences and identical phosphorylation sites described for protein kinase C and CaMPKII in several proteins^{20–22}.

Our results reveal that the persistent enhancement of peak I_{Ca} after periods of prolonged repetitive depolarization results from activation of CaMPKII triggered by an increase in $[\text{Ca}^{2+}]_i$. Such a mechanism is consistent with results showing that CaMPKII shifts calcium channels in inside-out excised patches of GH3 cells to a mode with very long openings²³, and that CaMPKII can phosphorylate the α -subunit of the L-type Ca^{2+} channel in skeletal muscle membranes²⁴. Furthermore, CaMPKII-mediated enhancement of I_{Ca} may help to explain why excitatory agonists such as acetylcholine trigger biphasic changes in both cytoplasmic Ca^{2+} concentration and contractile state^{25,26}. The first phase is transient, reflecting Ca^{2+} release from internal stores, and positive feedback of $[\text{Ca}^{2+}]_i$ on I_{Ca} may help explain the smaller but more sustained second phase, which is thought to be due to Ca^{2+} entry across the plasma membrane, presumably through voltage-activated Ca^{2+} channels. The central part played by CaMPKII in the positive feed-

back of Ca^{2+} on calcium channel activity fits nicely into this scheme as CaMPKII is localized to regions, in skeletal muscle at least, of the sarcoplasmic reticulum close to the cell membrane²⁷. □

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Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice

Masao Murakami*†, Takeshi Tsubata*, Masaya Okamoto*, Akira Shimizu‡, Shunichi Kumagai†, Hiroo Imura† & Tasuku Honjo*‡

* Department of Medical Chemistry and † Second Department of Internal Medicine, Faculty of Medicine, and ‡ Center for Molecular Biology and Genetics, Kyoto University, Sakyo-ku, Kyoto 606, Japan

STUDIES on transgenic mice expressing immunoglobulins against self-antigens^{1–6} have shown that self-tolerance is maintained by active elimination (clonal deletion)^{1–3,7}, functional inactivation (clonal anergy)^{4,5,8} of self-reactive B cells, or a combination of both⁶. We have established and characterized a transgenic mouse line expressing an anti-erythrocyte autoantibody⁶. In contrast to other autoantibody transgenic lines, about 50% of the animals of this transgenic line suffer from autoimmune disease, indicating a loss of self-tolerance. Here we show that peritoneal Ly-1 B cells (also known as B-1 cells⁹) are responsible for this autoimmune disease in our transgenic mice. A few self-reactive Ly-1 B cells that have somehow escaped the deletion mechanism expand in the peritoneum because of the absence of self-antigen. These Ly-1 B cells are eliminated *in vivo* by apoptosis once exposed to self-antigen. On the basis of these results we propose a novel autoantibody production mechanism whereby self-reactive B cells

sequestered in compartments free of self-antigens may survive, proliferate and be activated for generation of pathogenic autoantibodies in autoimmune diseases.

Ly-1 B cells, which are characterized by expression of surface antigens such as Ly-1⁺, IgM^{hi}, IgD^{lo}, B220^{lo} and Mac-1⁺, constitute a distinct B-cell population and differ in several of their functional properties from conventional B cells^{10,11}. Ly-1 B cells have a strong capacity for self-renewal¹² and are found predominantly in peripheral tissues such as the peritoneal^{10,11} and pleural cavities¹³. In transgenic mice that express an autoantibody against their red blood cells (RBC), normal numbers of Ly-1 B cells but not conventional B cells exist in the peritoneal cavity, regardless of any manifestation of autoimmune anaemia, and the number of B cells in the spleen and lymph nodes is dramatically reduced⁶.

First, we asked how peritoneal Ly-1 B cells escape from the deletion mechanism. To test whether the absence of the self-antigen, or RBC, allows Ly-1 B cells to survive in the peritoneum, we injected mouse RBC into the peritoneal cavity of 8-week-old transgenic and control mice. After a single injection of RBC, peritoneal Ly-1 B cells in transgenic mice were extinguished as early as 12 hours later and did not recover for at least 5 days, whereas the number of peritoneal B cells in the normal mice was unaffected by the same treatment (Fig. 1a). The peritoneal cells from the RBC-injected transgenic mice showed DNA fragmentation, a feature typical of apoptosis (programmed cell death)^{14,15}, but this was not seen in peritoneal cells from non-treated transgenic mice or RBC-injected normal mice (Fig. 1b), suggesting that apoptosis is induced by a specific interaction between anti-RBC IgM on Ly-1 B cells and mouse RBC. These results indicate that apoptosis is involved in clonal deletion of Ly-1 B cells by self-antigens *in vivo*.

The elimination of peritoneal Ly-1 B cells by self-antigen suggests that newly emerging Ly-1 B cells might be eliminated before homing to the peritoneal cavity in our transgenic mice. Indeed, we detected only a few B cells regardless of Ly-1⁺ in the peritoneum of 2-week-old transgenic mice and in the spleen of neonatal transgenic mice (Fig. 2a and b). By contrast, Ly-1 B cells constituted a large fraction (about 20%) of mononuclear

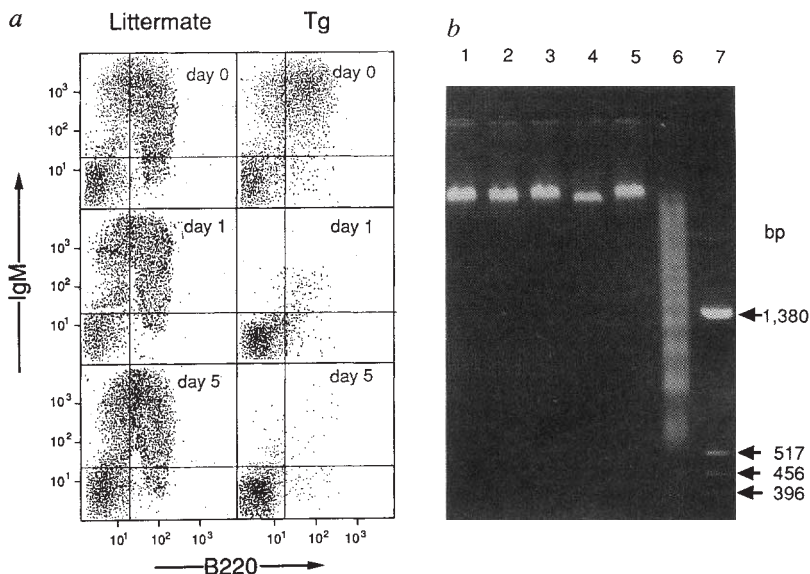
cells in both the neonatal spleen¹¹ and the 2-week-old peritoneum of normal mice (Fig. 2b). These results indicate that self-reactive Ly-1 B cells are actually eliminated *in vivo* early in the animal's life. A few self-reactive Ly-1 B cells that have somehow escaped clonal deletion and have migrated into the peritoneum are presumably able to proliferate in the absence of self-antigen.

Next we asked whether peritoneal Ly-1 B cells are responsible for the occurrence of haemolytic anaemia in our transgenic mice. We measured the number of anti-RBC autoantibody-producing cells in the spleen, bone marrow and peritoneum of the transgenic mice by enzyme-linked immunospot assay^{16,17}. No detectable anti-RBC autoantibody-producing cells were present in the spleen or bone marrow. The peritoneum of anaemic transgenics, however, contained a significant number of the autoantibody-producing cells, which correlated with the severity of the anaemia (Table 1). Autoantibody production was associated with peritoneal Ly-1 B cells fractionated by fluorescence activated cell sorting (data not shown). To investigate the role of peritoneal Ly-1 B cells in haemolytic anaemia in transgenic mice, we eliminated the peritoneal Ly-1 B cells of anaemic transgenic mice by injection of mouse RBC into the peritoneum every week. After 5 weeks, the haematocrit value recovered (Fig. 3a) and the amount of anti-RBC antibody bound to RBC significantly decreased (Fig. 3b). Peritoneal Ly-1 B cells are thus responsible for the haemolytic anaemia. This conclusion is also supported by our finding that transfer of peritoneal cells from anaemic transgenic mice causes anaemia in SCID mice¹⁸ (data not shown).

We have demonstrated that a small number of self-reactive Ly-1 B cells that have escaped the clonal deletion mechanism and have migrated into the peritoneum are able to survive and proliferate in the anti-RBC transgenic mice. In about half of the animals of this transgenic line, these peritoneal Ly-1 B cells produce autoantibody, which causes haemolytic anaemia. The expansion of the self-reactive Ly-1 B cells in the peritoneum is presumably attributable to the absence of self-antigen in the peritoneum, the self-renewal capacity of Ly-1 B cells and an environment in the peritoneum suitable for the growth of Ly-1

FIG. 1 Elimination of peritoneal Ly-1 B cells in transgenic (Tg) mice by an intraperitoneal injection of mouse RBC. a, Flow cytometric analysis of peritoneal cells of transgenic mice after RBC injection. Eight-week-old transgenics and normal littermates were injected intraperitoneally with 1×10^9 RBC in 500 μ l phosphate-buffered saline (PBS). After 1 or 5 days, mice were killed and their peritoneal cells collected. Cells were stained with antibodies and analysed. Cells from untreated mice were analysed as controls (day 0). b, DNA fragmentation analysis of cells from PBS- (lanes 1, 2) or RBC- (lanes 3–6) injected mice. Twelve hours after RBC injection, DNA from spleen cells (lanes 3, 5) and peritoneal cells (lanes 4, 6) from 6–8-week-old transgenic mice (lanes 5, 6) or normal littermates (lanes 3, 4) were analysed for DNA fragmentation. As controls, peritoneal cells from the PBS-injected normal littermates (lane 1) and transgenic mice (lane 2) were analysed in parallel. Molecular size standards (in base pairs) are *Hinf*I digests of Bluescript M13⁺ (lane 7).

METHODS. Transgenic mice are maintained as light- or heavy-chain gene heterozygotes as described⁶. Double-transgenic mice with the light- and heavy-chain genes were identified by Southern hybridization of tail DNA. Cells were stained with anti-B220 and anti-IgM antibodies and analysed by FACSscan (Becton Dickinson) as described⁶. Briefly, after blocking of Fc receptors on cells by excess mouse monoclonal IgG1 and IgG2b (Cappel), staining with rat anti-mouse B220(RA3-6B2) monoclonal antibody and biotinylated goat anti-mouse IgM antibody (Zymed) was followed by addition of fluorescein-conjugated mouse anti-rat kappa monoclonal antibody (Immunotec) and streptavidin-phycoerythrin conjugate (Becton Dickinson). Dead cells were excluded by propidium iodide staining. Macrophages, neutrophils and erythrocytes were gated out by forward and side scatter analysis. For the DNA fragmentation assay, 2×10^7 cells were lysed in



hypotonic buffer containing 10 mM EDTA, 200 mM NaCl, 0.1 mg ml⁻¹ proteinase K, 0.2% (v/v) Triton X-100 and 10 mM Tris-HCl, pH 7.5. DNA was extracted with phenol:chloroform (1:1, v/v) and then with chloroform:isoamyl alcohol (24:1, v/v), followed by precipitation with ethanol. Precipitates were resuspended in 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, incubated with 0.5 μ g ml⁻¹ RNase at 50 °C for 1 h and electrophoresed in a 2% agarose gel in 40 mM Tris-acetate, pH 8.0, containing 1 mM EDTA and 0.1 mg ml⁻¹ ethidium bromide; the gel was photographed under ultraviolet illumination.

TABLE 1 Localization of anti-RBC antibody-producing cells in transgenic mice

Mice	Number of mice tested	Frequency of anti-RBC antibody-producing cells ($\times 10^{-5}$)		
		Spleen	Bone marrow	Peritoneum
Transgenic mice with:				
severe anaemia	4	<10	<10	297 $\pm$ 94*
moderate anaemia	5	<10	<10	47 $\pm$ 15*
no anaemia	13	<10	<10	<10
Normal littermates	9	<10	<10	<10

Numbers of anti-RBC antibody-producing cells were measured by enzyme-linked immunospot assay as described previously^{14,15} with a slight modification. Plastic plates were coated with 100 μ l anti-idiotypic antibody (S54) against the anti-RBC autoantibody⁶ at the concentration of 10 μ g ml⁻¹ in PBS. After washing the plates with PBS, 1×10^5 cells were added to the wells and incubated for 12 h. After addition of 50 μ l per well of 5,000-times-diluted alkaline phosphatase-conjugated goat anti-IgM antibody (Tago), plates were washed and incubated with 100 μ l 1% agarose solution containing bromo-chloro-indolyl phosphate (Sigma) for 3 h. The number of spots was counted by microscopy ($n=3$). The transgenic mice were divided into three groups according to anaemia severity as indicated by haematocrit values. Mice having haematocrit values of <30% and of 30–40% are referred to as having severe and moderate anaemia, respectively; mice with haematocrits >40% are regarded as non-anaemic.

* Mean $\pm$ standard deviation.

B cells. A few conventional B cells could also migrate into the peritoneum, but they may not be able to proliferate because of their limited growth potential. For the development of haemolytic anaemia, activation of B cells is necessary. A wide variation in the severity of the anaemia in our transgenic mice⁶ suggests that environmental factors may be responsible for the activation of B cells.

Analysis of the sequences of IgG class autoantibodies derived from a single autoimmune-prone mouse has revealed that autoantibody-producing cells originate from a few clones^{19–21}. This suggests that expansion of self-reactive B cells may be

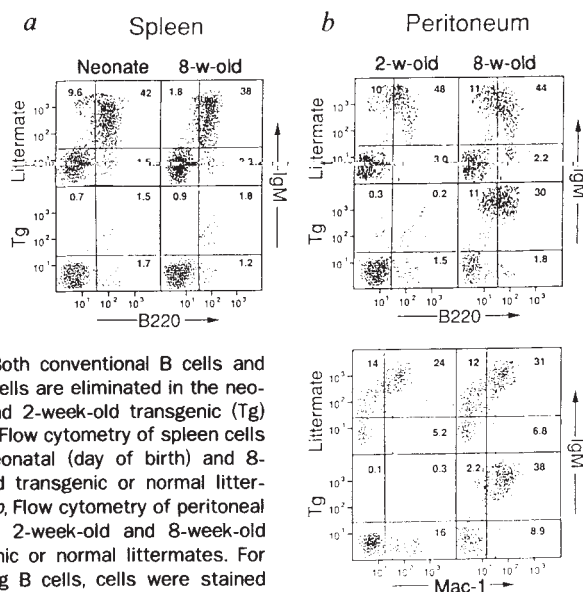


FIG. 2 Both conventional B cells and Ly-1 B cells are eliminated in the neonatal and 2-week-old transgenic (Tg) mice. *a*, Flow cytometry of spleen cells from neonatal (day of birth) and 8-week-old transgenic or normal littermates. *b*, Flow cytometry of peritoneal cells of 2-week-old and 8-week-old transgenic or normal littermates. For detecting B cells, cells were stained with anti-B220 monoclonal antibody and anti-mouse IgM antibody (Zymed). For detecting Ly-1 B cells in the peritoneum, cells were stained with anti-Mac-1 monoclonal antibody (M1/70.15.115.5HL) and anti-mouse IgM antibody. The percentage of positively stained cells is indicated in each compartment. More than 99% IgM positive cells expressed transgene-derived IgM on their surface as determined by staining with anti-idiotypic antibody (S54) against anti-RBC autoantibody⁶ (data not shown).

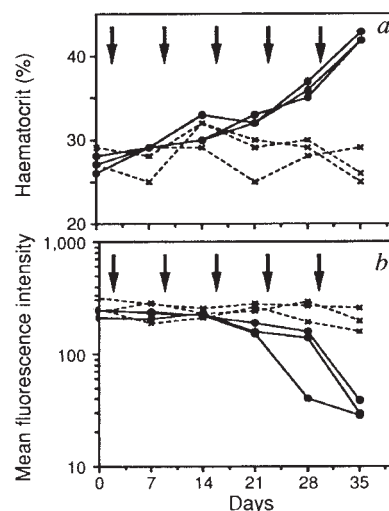


FIG. 3 Transgenic mice recover from anaemia after an intraperitoneal RBC injection. Mouse RBC (1×10^8 cells) were injected every 7 days into the peritoneum of three anaemic transgenic mice (6–8 weeks old) whose haematocrit values were less than 30% (solid lines with filled circles). As controls, similar anaemic transgenic mice were injected with PBS (broken lines with crosses). *a*, Time course of haematocrit values of transgenic mice injected with RBC or PBS. *b*, Time course of amount of autoantibody bound to circulating erythrocytes of the transgenic mice. Arrows indicate points of injection of RBC. For detecting antibody bound to RBC, circulating RBC were stained with fluorescein isothiocyanate-conjugated anti-IgM antibody (Cappel) and analysed by FACScan⁶. The mean fluorescence intensity of each sample was calculated. Haematocrits and mean fluorescence intensities in normal littermates are 45–50% and 1–10, respectively.

crucial for the pathogenesis of autoimmune disease. Our results indicate that self-reactive B cells, which should have been deleted in the presence of self-antigens, can proliferate in the peripheral tissues lacking the self-antigens. The association of Ly-1 B cells with autoantibody production^{22–24} may not be due to the genetically biased immunoglobulin variable-region gene repertoire of Ly-1 B cells, but rather to a strong capacity for self-renewal¹² which allows the self-reactive B cells to expand in the periphery without antigen stimulation. The generation and expansion of self-reactive Ly-1 B cells induced by this mechanism may be important in the pathogenesis of autoimmune diseases at the initial stage. Development of autoimmune diseases, however, may require affinity maturation, class switching, and terminal differentiation of self-reactive B cells, which are presumably induced by abnormalities of B and T lymphocytes and environmental factors^{25,26}. In fact the missing link is the absence of the Ly-1 marker on B cells producing IgG class autoantibodies with high affinities²⁷.

Apoptosis is involved in cell death induced by signalling through the cell-surface immunoglobulin of B-lymphoma cells^{28,29}. In our anti-RBC transgenic mice, peripheral self-reactive Ly-1 B cells expressing cell-surface IgM are eliminated by apoptosis *in vivo* when they encounter self-antigens (Fig. 1b). This result indicates that apoptosis may be involved in elimination of self-reactive B cells in the periphery of normal mice. The peripheral elimination of self-reactive B cells has also been demonstrated in transgenic mice in which B cells express cell-surface IgM and IgD reactive to the class I antigen of the major histocompatibility complex product³⁰. The peripheral deletion of B cells should be essential in eliminating self-reactive B cells that could easily be generated in the periphery from non-self-reactive B cells by somatic mutation^{31,32}.

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Molecular and biological characterization of a murine ligand for CD40

Richard J. Armitage*, William C. Fanslow*, Laura Strockbine†, Timothy A. Sato*, Ky N. Clifford*, Brian M. Macduff*, Dirk M. Anderson†, Steven D. Gimpel†, Terri Davis-Smith‡, Charles R. Maliszewski*, Edward A. Clark§, Craig A. Smith‡, Kenneth H. Grabstein*, David Cosman† & Melanie K. Spriggs†

Departments of *Immunology, †Molecular Biology and ‡Biochemistry, Immunex Research & Development Corporation, 51 University Street, Seattle, Washington 98101, USA

§Department of Microbiology SC-42, University of Washington, Seattle, Washington 98195, USA

THE CD40 surface molecule is a 277-amino-acid glycoprotein expressed on B lymphocytes, epithelial cells and some carcinoma cell lines. Monoclonal antibodies against CD40 mediate a variety of effects on B lymphocytes, including induction of intercellular adhesion^{1,2}, short- and long-term proliferation³⁻⁵, differentiation^{6,7} and enhanced tyrosine phosphorylation of proteins⁸. In addition, germinal centre centrocytes are prevented from undergoing apoptosis by activation through CD40 and receptor for antigen⁹. These data indicate that CD40 could be a receptor for an unknown ligand with important functions in B-cell development and activation. This hypothesis is strengthened by the homology of the extracellular region of the CD40 molecule¹⁰ with a family of cell-surface glycoproteins^{11,12} that includes the receptors for nerve growth factor^{13,14} and tumour necrosis factor^{11,15,16}. Here we report the cloning of a ligand for CD40 that is expressed on the cell surface of activated T cells and mediates B-cell proliferation in the absence of co-stimulus, as well as IgE production in the presence of interleukin-4.

We found previously that the murine thymoma cell line EL4 can specifically bind a biotin-labelled soluble fusion protein (CD40.Fc)¹⁷ consisting of the extracellular domain of CD40

linked to the Fc region of human IgG1. On the basis of this observation, EL4 cells were used to isolate the gene encoding the ligand for CD40 (CD40L).

EL4 cells were selected by repeated flow-cytometric cell sorting using biotinylated CD40.Fc. After five rounds of sorting, the level of CD40.Fc binding to the resulting cells (termed EL40.5) was significantly increased compared with that on unsorted EL4 cells (15,000 and 450 CD40.Fc binding sites per cell, respectively; data not shown). Messenger RNA was extracted from the sorted cells and used to construct a mammalian expression library as before¹⁸. A single complementary DNA clone, designated 19F, which was capable of binding radiolabelled CD40.Fc, was isolated. This clone contained an insert of ~1.5 kilobases (kb) with a short 5' noncoding region, a single open reading frame (ORF) of 780 nucleotides and ~700 nucleotides of 3' noncoding sequences. The predicted amino-acid sequence of the protein encoded by the ORF, based on the nucleotide sequence of this clone is shown in Fig. 1. The encoded polypeptide is 260 amino acids long, with a calculated unmodified relative molecular mass of 29,395. The amino-terminal 22 amino acids are followed by a 24-amino-acid stretch of hydrophobic residues, which presumably functions as a signal/anchor domain. This configuration is typical for a type-II transmembrane glycoprotein in which the C terminus of the protein is extracellular. The cell-surface form of tumour necrosis factor α (TNF α) is also a type-II glycoprotein¹⁹. There are four cysteine residues and a single potential N-linked glycosylation site in the putative extracellular domain of 214 amino acids. Comparison of both nucleotide and amino-acid sequences of this CD40L with the GenBank and EMBL databases²⁰ yielded no significant homology with any known proteins.

The surface expression of a ligand for CD40 on EL4 cells suggests a possible role for CD40L in cognate T-B cell interactions. To determine whether CD40L was expressed by functionally mature T cells, we analysed murine Th1 and Th2 T-cell clones²¹ by northern blotting to test for their ability to express CD40L mRNA upon activation with CD3 antibody (Fig. 2). A single band of ~2 kb was detected in the sorted EL40.5 cells and in the stimulated, but not unstimulated, T-helper cell lines. The size of this mRNA suggests that the 19F cDNA may be lacking noncoding sequences from the 5' and/or 3' end of the molecule.

The protein encoded by the 19F open reading frame was compared with CD40L from stimulated T-cell clones by ³⁵S-labelling and bioaffinity precipitation with CD40.Fc (Fig. 3). CV-1/EBNA cells transfected with the 19F ORF expressed a CD40-binding protein with M_r ~33,000. This protein comigrated with a CD40-binding protein in stimulated Th2 cells, but was not detectable in unstimulated Th2 cells, which is consistent with the observed level of expression of CD40L-specific mRNA (Fig. 2). The gels depicted in Fig. 3 were electrophoresed under reducing conditions. But immunoprecipitated sample analysed under non-reducing conditions were essentially identical (data not shown). It is still possible that the CD40L is a non-covalently linked dimer, given the probable dimeric nature of CD40 itself²⁴. The calculated M_r (29,395) of the unmodified CD40L, compared with the apparent M_r of 33,000 from Fig. 3, suggests that the N-linked glycosylation site in the extracellular domain may be used.

Radioiodinated CD40.Fc bound specifically to sorted EL40.5 cells with an association constant K_a ~ 2×10^9 M⁻¹ (data not shown). Direct binding assays of CV-1/EBNA cells transfected with the CD40L gene bound CD40.Fc with a comparable K_a of ~ 1×10^9 M⁻¹ (data not shown). Thus, naturally occurring and recombinant murine CD40L bind radiolabelled human CD40.Fc with similarly high affinity. The supernatant from CV1/EBNA cells transfected with clone 19F contained no detectable soluble CD40L as determined by labelling with ³⁵S and immunoprecipitation (data not shown). But the presence of two arginine residues in the extracellular domain, proximal

FIG. 1 Nucleotide and predicted amino-acid sequence of the ORF encoding a murine CD40L. The sequence of the putative signal/anchor domain is underlined. The protein has four cysteine residues (indicated by dots above the sequence) and a single putative *N*-linked glycosylation site (boxed).

METHODS. A cDNA library in the mammalian expression vector pDC406 was constructed essentially as described¹⁸. Pools of 2,000 cDNA clones were transfected into CV1/EBNA cells directly onto microscope slides¹⁸ and positive cells identified by their ability to bind ¹²⁵I-labelled CD40Fc.

The original positive pool was broken down into smaller pools until a single cDNA clone (19F), capable of binding radiolabelled CD40Fc, was isolated. This nucleotide sequence has been deposited at GenBank, accession number X65453.

to the transmembrane region of CD40L, provides a potential site for proteolytic cleavage²⁵, which could give rise to a soluble form of the ligand.

CV1/EBNA cells transfected with clone 19F, or with empty vector, were fixed with paraformaldehyde and examined for their ability to stimulate B-cell proliferation. Purified murine splenic B cells were induced to proliferate in a dose-dependent manner in the presence of CV1/EBNA cells transfected with CD40L, in the absence of co-stimulus (Fig. 4a). For comparison, 10 $\mu\text{g ml}^{-1}$ lipopolysaccharide induced $15,239 \pm 960$ c.p.m. in the same experiment (data not shown). Similarly, CV1/EBNA cells expressing CD40L were directly mitogenic for purified human tonsillar B cells (Fig. 4b). This stimulatory effect could be inhibited by the addition to cultures of soluble CD40Fc, but not a control Fc fusion protein, TNFRFc. In contrast, addition of soluble CD40 monoclonal antibody G28-5 at 200 ng ml^{-1} (a

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CTTTCAGTCAGCATGATAGAAACATACAGCCAACTTCCCCAGATCCGTGGCAACTGGACTTCCAGCGAGCATGAAGATTTTATGTATTTACTT 96
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TCTGTTCACTTGGCGGAGTGTTTGAATTAACAGCTGGTCTTCTGTGTTTGTCAACGTGACTGAAGCAAGCCAAGTATCCACAGAGTGGCTTC 768
SerValHisLeuGlyGlyValPheGluLeuGlnAlaGlyAlaSerValPheValAsnValThrGluAlaSerGlnValIleHisArgValGlyPhe 252
TCATCTTTTGCTTACTCAAACCTCTGAACAGTGCCTGTCTAGGCTGCA 818
SerSerPheGlyLeuLeuLysLeuEND 260

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concentration optimal for proliferation with co-mitogen) induced 768 ± 132 c.p.m. (data not shown). CV1/EBNA cells transfected with empty vector did not induce murine or human B-cell proliferation.

The ability of CD40L to induce IgE secretion was also investigated. In both murine (Fig. 4c) and human (Fig. 4d) systems, addition of fixed CV1/EBNA cells expressing CD40L induced IgE secretion from IL-4-stimulated B cells in a dose-dependent manner. By comparison, in the experiment detailed in Fig. 4c, 10 $\mu\text{g ml}^{-1}$ lipopolysaccharide and 1×10^6 fixed CD3 antibody-activated murine Th2 cells induced $2,661 \pm 131$ and $2,540 \pm 231$ ng ml^{-1} of murine IgE, respectively (data not shown). Addition of 500 ng ml^{-1} soluble G28-5 in the experiment detailed in Fig. 4d induced 29 ng ml^{-1} human IgE (data not shown). The response of human B cells was specifically inhibited by addition of CD40Fc to cultures (Fig. 4d). CV1/EBNA cells transfected

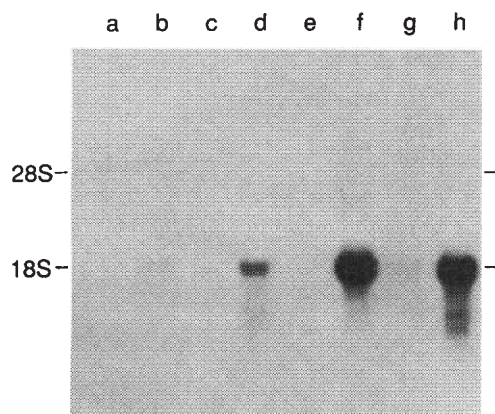


FIG. 2 Northern blot analysis of murine T-cell clones expressing the CD40L mRNA. RNA was isolated from unsorted EL4 cells (lane a) or sorted EL40.5 cells (lane b), and electrophoresed alongside RNA isolated from murine 7C2 (lanes c, d), 7F9 (lanes e, f), and 4G1 (lanes g, h) T-helper cell clones. RNA from cells stimulated with CD3 monoclonal antibody is shown in lanes d, f, h. Lanes c, e and g, represent RNA extracted in parallel from unstimulated cells. Migration of ribosomal RNA marks is shown on the left.

METHODS. Total RNA was extracted from 7C2 (Th1), 7F9 (Th2) and 4G1 (Th2) cell clones cultured for 4 h in the presence or absence of 1 $\mu\text{g ml}^{-1}$ of immobilized CD3 antibody 2C11 (ref. 22). RNA was electrophoresed on 1% agarose/formaldehyde gels alongside total RNA isolated from the unsorted EL4 cell line and from sorted EL40.5 cells from which the expression library was derived. RNA was transferred to nitrocellulose and probed with a ³²P-labelled antisense RNA probe corresponding to the sequence shown in Fig. 1 as described²³.

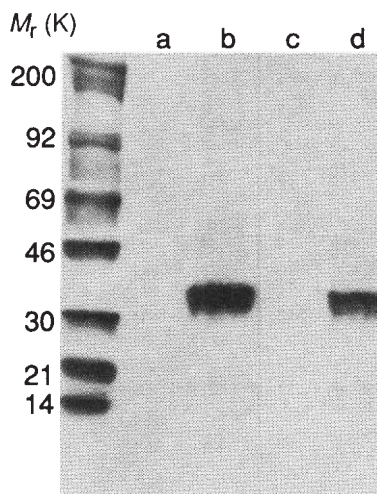


FIG. 3 SDS-PAGE analysis of immunoprecipitated CD40L protein. Lane a, CV1/EBNA cells transfected with empty vector; lane b, CV1/EBNA cells transfected with CD40L; lane c, unstimulated Th2 cells; lane d, stimulated Th2 cells. Size markers are in the left lane.

METHODS. CV1/EBNA cells were transfected with clone 19F as described¹⁸. Three days post-transfection, cells were radiolabelled with 100 $\mu\text{Ci ml}^{-1}$ of ³⁵S TRANS-label in parallel with 7F9 Th2 cells stimulated with 1 $\mu\text{g ml}^{-1}$ of immobilized CD3 antibody 2C11 (ref. 22) for 6 h. Lysates were prepared²³ and immunoprecipitated with 5 $\mu\text{g ml}^{-1}$ CD40Fc followed by protein A-Sepharose. Immunoprecipitates were analysed on 8–16% SDS-polyacrylamide gels under reducing conditions.

with empty vector had no effect in either assay system. In the absence of IL-4, cells expressing CD40L induced no detectable murine or human IgE (data not shown).

The biological activities of CD40L in both proliferation and IgE secretion assays fulfill predictions made for the functional effects of the ligand based on the previously described activity of CD40 monoclonal antibody³⁻⁷. Such activity is not species-restricted, as murine CD40L binds to human CD40 with high affinity and is active on human B cells. The directly mitogenic nature of the ligand in the absence of co-stimulus points to the use of a novel pathway of signal transduction distinct from that invoked by binding of cytokines such as the interleukins IL-1,

IL-2, IL-4, IL-5 and IL-6, which are mitogenic only for preactivated B cells. The mitogenic activity of CD40L is likely to be dependent on its expression as a cell-membrane molecule as immobilized CD40 antibody provides a similar proliferative signal⁵ which is not seen with soluble antibody.

Signalling through CD40 results in the formation of heterotypic adhesions between T and B cells², and the enhancement of expression of intercellular adhesion molecule-1 (ICAM-1, CD54)², a ligand for leucocyte function antigen-1 (LFA-1, CD11a/CD18). This, together with the failure to detect soluble recombinant CD40L in the supernatant of transfected CV1/EBNA cells, suggests an important functional rule for CD40L as a membrane-anchored protein. This is further supported by the greatly enhanced levels of surface ligand expression observed when T cells are activated through the T-cell receptor-CD3 complex.

The combination of membrane-bound CD40L and the potential for a soluble form of ligand may provide a mechanism by which T cells can regulate B-cell expansion and differentiation depending on the form in which the ligand is presented to its receptor. Expression of soluble constructs of the cloned murine CD40L will allow a comparison of their biological effects to be made with those demonstrated for membrane-bound ligand. Furthermore, cloning of the human homologue will enable elucidation of the range of biological activities regulated by CD40L, a molecule which is important in B-cell responses. □

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Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein

P. Renee Yew & Arnold J. Berk

Department of Microbiology and Molecular Genetics and the Molecular Biology Institute, University of California, Los Angeles, California 90024-1570, USA

THE cellular phosphoprotein p53 inhibits progression through the mammalian cell cycle^{1,2}. Both p53 alleles are frequently mutated in human tumours^{3,4}, indicating that p53 is a tumour suppressor. Recent studies have suggested that p53 functions as a transcriptional activator⁵⁻⁸, but the significance of this activity in cell-cycle control has not been established. The adenovirus 2 (Ad2) early 1B (E1B) 55K protein binds to p53 in transformed cells⁹ and con-

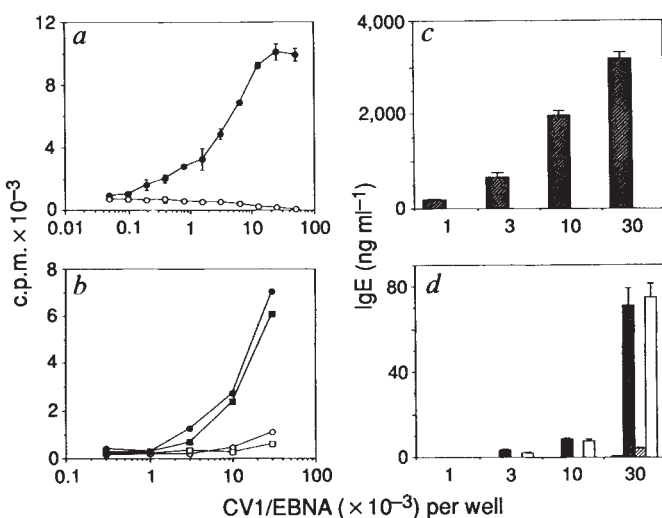
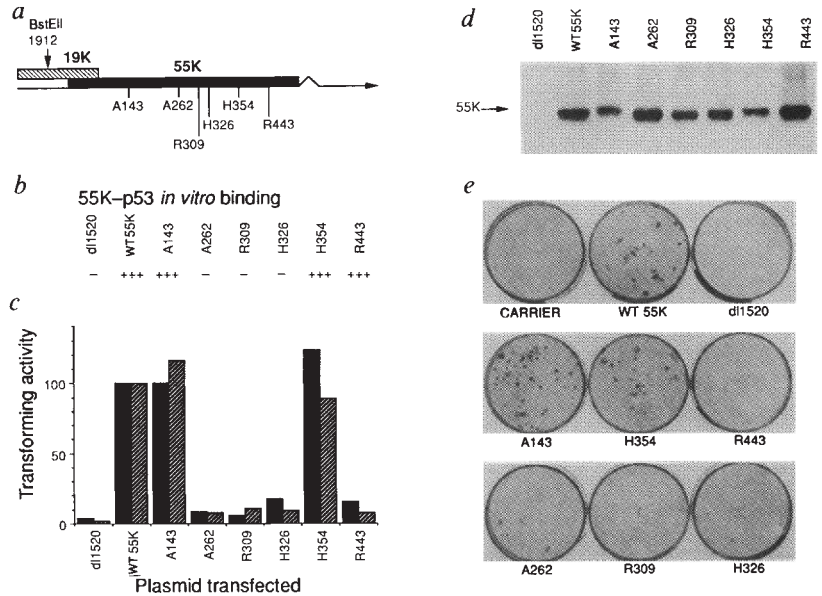


FIG. 4 Murine CD40L has activity on murine and human B cells. *a*, Proliferative response of purified murine splenic B cells was determined after culture for 4 days with a titration of fixed CV1/EBNA cells transfected with CD40L (●) or empty vector (○). Results are expressed as mean c.p.m. $\pm$ s.e.m. of triplicate cultures. *b*, Proliferative response of purified human tonsillar B cells was determined after culture for 7 days with a titration of fixed CV1/EBNA cells expressing CD40L, alone (●), or in the presence of 25 μ g ml⁻¹ CD40.Fc (□) or 25 μ g ml⁻¹ TNFR.Fc (■). Control cultures were run with CV1/EBNA cells transfected with empty vector (○). Results are expressed as mean c.p.m. of triplicate cultures. *c*, Secreted IgE was determined from supernatants of purified murine splenic B cells cultured for 7 days in the presence of 10 ng ml⁻¹ recombinant murine IL-4 and a titration of fixed CV1/EBNA cells transfected with CD40L. *d*, Secreted IgE was determined from supernatants of human T-depleted peripheral blood mononuclear cells cultured for 10 days in the presence of 5 ng ml⁻¹ recombinant human IL-4 and a titration of fixed CV1/EBNA cells transfected with CD40L, alone (heavy ▒), or in the presence of 25 μ g ml⁻¹ CD40.Fc (light ▒) or 25 μ g ml⁻¹ TNFR.Fc (■). Control cultures contained CV1/EBNA cells transfected with empty vector (●). Results are expressed as mean ng ml⁻¹ $\pm$ s.e.m. of triplicate cultures.

METHODS. CV1/EBNA cells were fixed at 3 days post-transfection in 1% paraformaldehyde for 5 min at 25 °C. For proliferation assays, murine B cells (1×10^5 per well) were cultured in flat-bottomed 96-well microtitre plates in 200 μ l RPMI in a humidified atmosphere of 10% CO₂. Medium was supplemented with 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 2 mM L-glutamine and 50 μ M 2-mercaptoethanol. Human B cells (1×10^5 per well) were cultured in flat-bottomed 96-well microtitre plates in RPMI in a humidified atmosphere of 10% CO₂. Medium was supplemented with 10% FBS, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin. Wells were pulsed with 2 μ Ci ³H-thymidine (25 Ci mmol⁻¹) for the final 8 h of culture, cells collected, and c.p.m. incorporated determined by liquid scintillation spectrometry. For the determination of murine IgE secretion, culture media were supplemented with RPMI 1640 as described for murine B-cell proliferation assays, but containing 20% FBS. Cultures (2 ml) were set up in triplicate and contained 1×10^6 purified B cells. Culture conditions for the determination of human IgE were as described for human B-cell proliferation assay except that round-bottomed 96-well microtitre plates were used. IgE in culture supernatants was determined by standard enzyme-linked immunosorbent assay sensitive to 100 pg ml⁻¹ and 4 ng ml⁻¹ human and murine IgE, respectively.

FIG. 1 *a*, Diagram of the E1B messenger RNA (sedimentation coefficient, 22S) showing the open reading frames encoding the 19K and 55K proteins and the six 55K insertion mutations¹⁴. Mutations are named by a letter representing the restriction site at which a 12-base-pair (bp) linker was inserted and a number indicating the amino-acid residue at or just preceding the 4-residue insertion. Expression of the 19K protein was eliminated by cutting at the 1,912-bp *Bst*Ell restriction site, filling in the overhang, and religating the plasmid to introduce 5 bp. *b*, *In vitro* binding of 55K and p53 proteins¹⁴: a minus sign represents no binding and plus signs strong binding. *c*, Results of two independent BRK focus assays with plasmid lacking expression of the 19K protein. Focus-forming activity is represented as a per cent of wild-type activity. Wild-type (WT) 55K protein represents Ad2; dl1520 is a deletion mutant expressing no 55K protein¹⁰ and indicates the level of transformation by E1A alone. *d*, Immunoblot showing the steady-state level of 55K protein in infected BRK cell extracts probed with the 2A6 anti-55K protein monoclonal antibody²¹ with WT 55K protein represented by Ad2. *e*, Representative crystal-violet-stained plates showing foci formed from transformed primary BRK cells. Carrier DNA used was salmon sperm DNA and WT 55K protein was Ad2.

METHODS. Generation of primary BRK cells, the BRK cell-transformation assay, and crystal violet staining of plates have all been described¹⁴. The average number of foci per plate for WT 55K was 65 in experiment 1, and 57 in experiment 2. Three 60-mm plates were transfected for each sample in each experiment. Immunoblotting: BRK cells were plated at a density of 1×10^6 cells per 60-mm plate and infected at a multiplicity of infection of 50 with adenovirus containing the wild-type or mutant genes encoding the 55K proteins¹⁴. Cells were collected 27 h



postinfection by lysis in 100 μ l 1% Triton X-100, 0.05% SDS, 10 mM Tris-HCl, pH 8, 0.15 M NaCl and 1 mM EDTA, and one third of the lysate was analysed by SDS-PAGE. Proteins were transferred to nitrocellulose and incubated with monoclonal 2A6 hybridoma supernatant against the 55K protein. The signal was detected as described²².

tributes to oncogenic transformation by Ad2 (refs 10–12). Here we report that mutants of E1B 55K and wild-type Ad12 E1B 54K proteins show a strong correlation between their ability to inhibit p53-mediated transcriptional activation and their ability to cooperate with adenovirus E1A protein in the transformation of primary cells. These results indicate that p53 probably inhibits cell cycling by functioning as a transcription factor.

Six mutants of the adenovirus 2 E1B 55K protein (relative molecular mass, 55,000) with four amino-acid in-frame insertions (Fig. 1a) were assayed for their ability to transform primary baby rat kidney (BRK) cells in cooperation with E1A, to bind p53 and to inhibit p53-mediated transcriptional activation. Mutants A143, H354 and R443 bind p53 *in vitro* like the wild-type 55K protein, but mutants A262, R309 and H326 do not bind p53 (ref. 13, Fig. 1b). Another mutant¹⁰, dl1520, does not express any 55K protein. To reveal the effects of mutations in the 55K protein on BRK transformation, it was necessary to

eliminate expression of the E1B 19K protein (Fig. 1a) because this protein alone can cooperate with E1A in a focus assay on BRK cells¹². Each of the mutant proteins accumulated to levels comparable with 55K wild-type protein in BRK cells (Fig. 1d).

The results of our transformation assay (Fig. 1c, e) fit well with a model in which binding of 55K protein to p53 inactivates p53 function, reducing controls on cell cycling and favouring transformation^{1,2}. But mutant R443 is a notable exception to this correlation: its transforming activity was severely reduced even though it bound like the wild-type protein to p53 in an *in vitro* assay¹⁴. To determine whether this inconsistency might be explained by a failure of R443 to bind p53 *in vivo*, we subjected extracts from metabolically labelled, infected BRK cells to a double-immunoprecipitation protocol. Immunoprecipitates prepared with a monoclonal antibody against the 55K protein were dissociated and reprecipitated with a monoclonal antibody against p53. On the basis of these results (Fig. 2a), we conclude

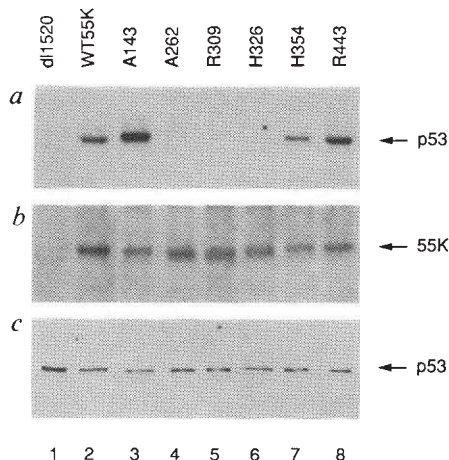


FIG. 2 *In vivo* 55K-p53 protein binding. *a*, Double immunoprecipitation with anti-55K followed by anti-p53 antibody showing co-immunoprecipitated, labelled p53 protein. Immunoblots of extracts used for the double

immunoprecipitation experiment with anti-55K antibody (*b*) and anti-p53 antibody (*c*). Wild-type 55K protein was from Ad2 and mutations are listed as they appear in the 55K polypeptide from amino to carboxy terminus. **METHODS.** Extracts for the double immunoprecipitation were generated by infecting primary BRK cells at a multiplicity of infection of 50 and labelling cells 24 h postinfection for 5.5 h with 150 μ Ci [³⁵S] translabel (ICN, Inc.) per 60-mm plate in methionine-free medium. Cells were harvested by lysis in RIPA buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.15 M NaCl, 1% NP40, 1% sodium deoxycholate and 1 mM PMSF) and 2×10^7 c.p.m. of material precipitated with trichloroacetic acid were incubated with 250 μ l hybridoma supernatant containing 2A6 anti-55K monoclonal antibody. The immunoprecipitate was recovered using protein A-Sepharose and washed twice with RIPA buffer. The immunoprecipitate was then dissociated from the Sepharose by boiling in 25 μ l 3% SDS, 93 mM Tris, pH 6.8, 15% glycerol, 1.1 M β -mercaptoethanol and 0.03% bromophenol blue. The protein A-Sepharose beads were removed by centrifugation and the supernatant was diluted 25-fold and re-immunoprecipitated with 250 μ l hybridoma supernatant containing pAb421 anti-p53 monoclonal antibody²³. The immunoprecipitate was analysed by SDS-PAGE, the gel was fixed and treated with 1 M sodium salicylate for fluorography, and autoradiographed. The ³⁵S-labelled infected extracts were immunoblotted using the same number of trichloroacetic acid-precipitable counts per sample and probing the immunoblots with 2A6 anti-55K or pAb240 anti-p53 (ref. 24) antibodies as described in the legend to Fig. 1.

that the R443 mutant does bind p53 *in vivo* but is defective in 55K transforming activity.

The acidic amino terminus of p53 functions as a transcriptional activation domain when fused to the DNA-binding domain of the yeast transcription factor GAL4 (refs 5–7). The adenovirus E1B 55K protein binds to this same amino-terminal region of p53 (ref. 13). If p53 inhibits cell cycling by acting as a transcriptional activator of cell-cycle negative-regulatory genes, then the 55K protein might function by inhibiting p53-mediated transcriptional activation. We therefore analysed the ability of wild-type and mutant 55K proteins to inhibit GAL4-p53 transactivation in a transient transfection assay (Fig. 3a), knowing that 55K bound to GAL4-p53 translated *in vitro* (data not shown).

As shown previously⁷, the carboxy-terminus of p53 had no transcriptional activity, although full-length p53 in the presence of K55 (a control expression vector that does not express the

55K protein) gave substantial activation (Fig. 3b). When E1B 55K protein was coexpressed, activation by GAL4-p53 was drastically reduced. Activation by GAL4-VP16 (Fig. 3b) or GAL4-E1A (ref. 15) (data not shown) was not affected by the 55K protein, demonstrating that its inhibition of GAL4-p53 was specific. We observed similar results in COS and NIH3T3 cells (data not shown).

As expected, the mutants A262, R309 and H326 that do not bind p53 do not inhibit p53 transcriptional activation (Fig. 3b), but mutants A143 and H354, which bind p53 and transform BRK cells, greatly reduce activation by GAL4-p53. Mutant R443, which binds p53 but is defective in transformation, only slightly reduces activation by GAL4-p53. With the exception of R309, differences in inhibition of p53 transactivation by these mutants could not be explained by differences in expression of mutant proteins (Fig. 3c).

Wild-type murine p53 protein can activate transcription from the murine muscle-specific creatine kinase promoter in a transient transfection assay⁸. It is likely that the transcription control region of this creatine kinase gene contains binding sites for the p53 DNA-binding domain¹⁶, and we find that adenovirus E1B 55K protein also inhibits activation of the creatine kinase promoter by wild-type p53 (Fig. 4a). Moreover, each of the

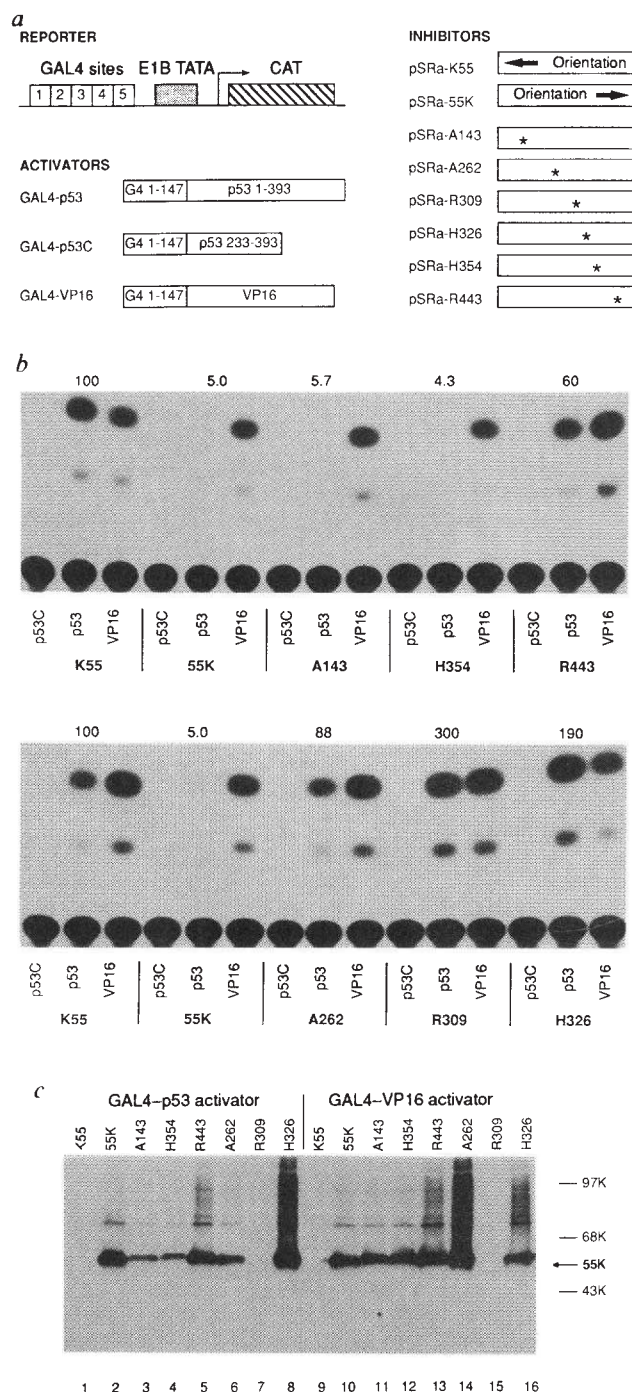
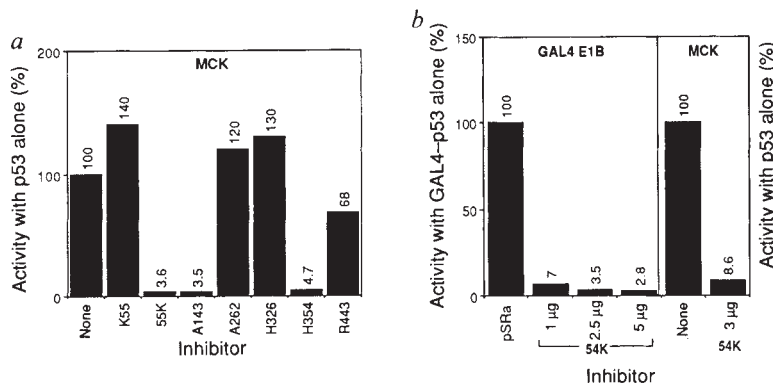


FIG. 3 Inhibition of GAL4-p53 transactivation by 55K protein. **a**, DNA constructs used in co-transfection for chloramphenicol acetyl transferase (CAT) assays showing the reporter, activator and inhibitor plasmids; pSRa- refers to the pcDL-SRα296 vector²⁵ used to express wild-type and mutant 55K proteins. Asterisks denote the sites of four amino-acid insertions in the 55K mutants. pSRa-K55 contains an insertion of the coding region for 55K protein in the antisense orientation in pcDL-SRα296. Expression vectors for GAL4 fusions to full-length p53, the C-terminal 160 residues of p53, or the activation domain of herpes simplex virus activator VP16 were transfected into HeLa cells with the target gene (five GAL4 binding sites and the E1B TATA box controlling the CAT gene) and the plasmids expressing wild-type or mutant 55K. As a control, a vector carrying the wild-type 55K gene inserted in the reverse orientation (K55) was also used. **b**, Thin-layer chromatography (TLC) showing CAT assay results, where each 55K sample was tested with GAL4 fusions to activators p53C (C terminus), p53 full-length or VP16. The two rows show samples A143, H354 and R443 in the top panel compared with controls K55 and 55K in the top panel. The bottom panel shows samples A262, R309 and H326 compared with the K55 and 55K controls in the bottom panel. Activation was quantitated by scanning the entire TLC plate using the AMBIS β-scanning system: the level of p53 activation was normalized to the per cent of GAL4-p53 activation in the presence of the appropriate K55 control. Values shown above each GAL4-p53 sample represent an average of three independent CAT assay transfections for each sample, except in the case of the 55K sample, which is an average of four transfections, and R309, which is an average of two transfections. Different amounts of the pcDL-SRα296 55K expression plasmids were transfected to obtain comparable levels of 55K protein for the different 55K mutants. These amounts were 1 μg DNA per 60-mm plate for K55 (top panel), 55K, A143; 0.5 μg DNA per plate for H354 and R443; 7 μg DNA per plate for A262; and 10 μg per plate for K55 (bottom panel), R309 and H326. **c**, Immunoblot using 55K antibody to the GAL4-p53 (left) and the GAL4-VP16 (right) co-transfected extracts analysed in the CAT assay.

METHODS. The 55K gene was cloned into the *Pst*I site of the pcDL-SRα296 expression vector²⁵ as a 1,820-bp *Pst*I partial fragment from the pUC19.R-/Ad1767-3632 plasmid¹⁴. The 55K mutants were cloned as *Bst*EII (Ad1912)-*Sst*I (Ad3632) fragments into the pcDL-SRα296-55K plasmid, replacing the wild-type sequences. HeLa cells were transfected by calcium phosphate¹⁴ using 0.5 μg GAL4-p53, 0.5 μg GAL4-p53C or 0.1 μg GAL4-VP16 plasmids per 60-mm plate; 1 μg per plate of reporter plasmid, 1 μg per plate of the β-galactosidase expression plasmid, pCH110 (ref. 26), and the indicated amounts of inhibitor expression vectors or K55 control. Total DNA in the transfection was brought to 10 μg per plate by adding salmon sperm DNA. All samples were normalized for transfection efficiency by measuring β-galactosidase activity. Samples ranged between 9 to 30 β-galactosidase units. CAT was assayed as described²⁷ using ~20 to 50 μl GAL4-p53 and GAL4-p53C cell extracts and 1% of this volume for GAL4-VP16 samples. CAT assay samples were immunoblotted by normalizing the amount of extract used according to β-galactosidase activity and probing the immunoblot with 2A6 anti-55K antibody as described in Fig. 1 legend.

FIG. 4 Ad2 55K and Ad12 54K inhibition of p53 transactivation.

a, Ad2 55K inhibition of murine p53 transactivation of the muscle-specific creatine kinase (MCK) promoter. CAT activity is represented as a per cent of the activity with murine p53 alone. Reporter plasmid p3300 MCK CAT contains 3,300 bp of the murine MCK promoter upstream of the CAT coding region.⁸ The effector plasmid, p11-4, expressed wild-type full-length murine p53 (ref. 28). Inhibitor plasmids are shown in Fig. 3a. The amount of inhibitor plasmid transfected per 60-mm plate was varied to equalize expression of the different 55K mutants as assayed by immunoblotting (1 µg plasmids K55, 55K, A143 and H354; 8 µg plasmids A262 and H326; and 0.7 µg plasmid R443). Values shown are averages of three (K55, H326, R443) or four (55K, A143, A262, H354) independent transfection experiments. **b**, Ad12 54K inhibition. CAT assay results showing the inhibitory effect of Ad12 54K on GAL-p53 activation of the GAL4 E1B promoter (left) and on murine p53 activation of the MCK promoter (right). CAT activity is expressed as a per cent of the CAT activity observed without inhibitors. The Ad12 54K expression plasmid contains the 1,737-bp *EaeI* to *BstEII* fragment cloned into pSRα; all other plasmids are described in **a** and in Fig. 3. Values shown are averages of two independent CAT assay transfections for all samples. The amount of DNA transfected per 60-mm plate was 5 µg for pSRα and 1 µg, 2.5 µg, 3 µg or 5 µg (as indicated) for Ad12 54K. Ad12 54K co-expression with the GAL4-VP16 effector had no



adenovirus mutant proteins influences activation by wild-type p53 in this system in a manner analogous to their effects on activation by the GAL4-p53 fusion protein (Fig. 4a).

When the 55K protein of adenovirus 2 binds p53 in BRK cells, the complex is sequestered in the cytoplasm¹⁷. Perhaps the 55K protein's inhibition of transcriptional activation by p53 resulted from this cytoplasmic sequestration rather than being directly related to the function of p53 in the control of cell cycling. To address this point, we studied the related 54K protein encoded in the E1B region of adenovirus 12. Like Ad2 55K, Ad12 54K protein contributes to transformation of primary cells¹⁸ but does not sequester p53 in the cytoplasm¹⁹. We reasoned that if these two adenovirus E1B proteins function analogously in the transformation process and if inhibition of transactivation by p53 is necessary for transformation, then Ad12 54K should also inhibit activation by GAL4-p53, which was indeed the case (Fig. 4b). Adenovirus 12 54K protein also inhibited activation of the murine muscle-specific creatine kinase promoter by wild-type murine p53 (Fig. 4b). Consequently, cytoplasmic sequestration is not required for inhibition of p53 transactivation by the Ad12 54K protein.

These results show that the ability of adenovirus E1B proteins to inhibit p53 transactivation correlates strongly with their ability to cooperate with E1A in the transformation of primary cells. The correlation extends to the Ad2 E1B 55K mutant R443. Even though this mutant binds p53 *in vivo* (Fig. 2), it has a greatly reduced ability to inhibit p53 transactivation (Fig. 3 and 4a) and to transform BRK cells (Fig. 1). This correlation also extends to Ad12 54K protein, which does not form complexes with p53 that are sufficiently stable to be immunoprecipitated^{19,20} but still inhibits p53 transactivation (Fig. 4b) and functions in Ad12 transformation¹⁸. The fact that these adenovirus transforming proteins inhibit p53 transactivation strongly supports a model in which p53 inhibits cell cycling through its function as a transcription factor. □

inhibitory effect on VP16 activation (data not shown).

METHODS. Transfections into CV-1 cells for the MCK promoter and CAT assays are described in Fig. 3 legend. DNA transfected was 6 µg per 60-mm plate for p3300 MCK CAT, and 3 µg per plate for p11-4. The pSRα-Ad12 54K expression plasmid was constructed by filling in the 1,737-bp *EaeI* to *BstEII* fragment from plasmid pHAB6 (ref. 29), adding *EcoRI* linkers, and ligating into the pSRα plasmid.

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Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations

Edward Arnold*, Alfredo Jacobo-Molina*, Raymond G. Nanni*, Roger L. Williams*, Xiaode Lu*, Jianping Ding*, Arthur D. Clark Jr*, Anqiang Zhang*, Andrea L. Ferris†, Patrick Clark‡, Amnon Hizi§ & Stephen H. Hughes†

* Center for Advanced Biotechnology and Medicine (CABM) and Rutgers University Chemistry Department, 679 Hoes Lane, Piscataway, New Jersey 08854-5638, USA

‡ Program Resources Inc. and † ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, PO Box B, Frederick, Maryland 21702-1201, USA

§ Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69778, Israel

AIDS, caused by human immunodeficiency virus (HIV), is one of the world's most serious health problems, with current protocols being inadequate for either prevention or successful long-term treatment. In retroviruses such as HIV, the enzyme reverse transcriptase copies the single-stranded RNA genome into double-stranded DNA that is then integrated into the chromosomes of

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TABLE 1 Summary of crystallographic data collection and phasing

(a) Ternary complex of HIV-1 reverse transcriptase p66/p51 with Fab28 and 19/18 dsDNA
Mass of asymmetric unit = 178K

Data collection statistics

Dataset	Number of crystals	Unique reflections	Completeness (%)	R_{merge}^*	$R_{\text{deriv}}^\dagger$
Native	2	5,211	93.9	0.05	—
TAMM	1	5,129	92.4	0.07	0.13
PIP	1	5,233	94.3	0.08	0.20
Hg-UTP	1	5,356	96.5	0.06	0.17

Isomorphous replacement phasing statistics

Dataset	Number of sites	Site	Heavy atom parameters				$R_{\text{centric}}^\ddagger$	Phasing power§
			x	y	z	occ		
TAMM	3	Hg-A	0.261	0.367	0.159	0.47	0.55	1.98
		Hg-B	0.499	0.671	0.001	1.03		
		Hg-C	0.242	0.370	0.130	0.44		
PIP	7	Pt-A	0.103	0.612	0.071	1.12	0.59	1.81
		Pt-B	0.399	0.525	0.066	1.06		
		Pt-C	0.466	0.580	0.152	0.75		
		Pt-D	0.646	0.740	0.158	0.73		
		Pt-E	0.566	0.457	0.031	0.63		
		Pt-F	0.629	0.610	0.096	0.56		
		Pt-G	0.232	0.446	0.148	0.51		
Hg-UTP	4	Hg-A	0.267	0.368	0.153	0.78	0.57	1.76
		Hg-B	0.500	0.671	0.002	1.35		
		Hg-D	0.635	0.694	0.103	0.46		
		Hg-E	0.631	0.668	0.125	0.31		

The overall MIR figure-of-merit (FOM) was 0.672 for 4,482 reflections with $F > 3\sigma(F)$ between 25 and 7 Å.

The final FOM after iterative solvent flattening was 0.899 for 4,482 reflections.

(b) Complex of HIV-1 reverse transcriptase p66/p51 with Fab28 not containing DNA
Mass of asymmetric unit = 166K

Data collection statistics

Dataset	Number of crystals	Unique reflections	Completeness (%)	R_{merge}^*	$R_{\text{deriv}}^\dagger$
Native	2	5,212	93.9	0.07	—
TAMM	2	5,539	99.8	0.07	0.15
PIP	2	5,286	95.2	0.08	0.21

Isomorphous replacement phasing statistics

Dataset	Number of sites	Site	Heavy atom parameters				$R_{\text{centric}}^\ddagger$	Phasing power§
			x	y	z	occ		
TAMM	2	Hg-A	0.262	0.368	0.157	1.67	0.66	1.49
		Hg-B	0.503	0.670	0.003	0.86		
PIP	5	Pt-A	0.106	0.613	0.072	1.13	0.67	1.43
		Pt-B	0.403	0.529	0.066	1.24		
		Pt-C	0.480	0.587	0.149	0.61		
		Pt-D	0.644	0.748	0.158	0.95		
		Pt-H	0.648	0.677	0.091	0.92		

The overall MIR FOM was 0.478 for 5,173 reflections with $F > 2\sigma(F)$ between 25 and 7 Å.

The final FOM after iterative solvent flattening was 0.843 for 5,173 reflections.

Components were prepared and crystals were grown as previously described¹⁶. The oligonucleotides were synthesized in the CABM DNA Synthesis Network Laboratory (M. Leibowitz). The two crystal forms are isomorphous, belong to space group $P3_212$, with $a = 168.7$ Å and $c = 220.3$ Å, and contain one complex per asymmetric unit. Here we have used a mutant of HIV-1 reverse transcriptase (Cys 280 to Ser) that is fully active in polymerization and has RNase H activity that is resistant to oxidation¹⁷. All data used for the electron density calculations were recorded on a Xuong-Hamlin Mark II electronic area detector system (San Diego Multiwire Systems) mounted on a Rigaku RU-200 rotating anode X-ray generator (Cu K α , 0.3 mm focal spot, 5.4 kW). A typical crystal measured 1.0 mm $\times$ 0.6 mm $\times$ 0.4 mm and had a useful exposure lifetime (intensity decay less than 20%) of 10–20 h at -10°C . Isomorphous scaling of derivative to native intensity datasets, heavy atom refinement, and density modification through solvent flattening¹⁸ were done using the PHASES program package¹⁹. Derivatives are designated as: TAMM for tetrakis(acetoxymethyl)mercuric methanediol (Strem Chemicals); PIP for di- μ -iodobis(ethylenediamine)-di-platinum (II) nitrate (Strem Chemicals); and Hg-UTP for 5-mercuriuridine 5'-triphosphate (Sigma). Complete three-dimensional solutions for heavy-atom isomorphous difference Patterson syntheses were generated using both minimum and sum functions in the program VECTORMAP (R.L.W. and E.A., unpublished results; available from the authors). Common sites in related derivatives are given the same designations (for example, the Hg-A site is present in both TAMM and Hg-UTP). Initial solution of the heavy-atom derivative sites were provided by analyses of 2 mM TAMM and 1 mM $\text{Nb}_6\text{Cl}_{14}$ (obtained from D. Cascio, UCLA) derivatives of the non-DNA-containing crystals using data between 25 and 8 Å resolution (the $\text{Nb}_6\text{Cl}_{14}$ sites refined poorly and were not used in subsequent calculations). Cross-phased difference Fourier syntheses gave a clear verification of the major sites and revealed additional sites that were initially confirmed using cross-vector searches with known sites as input into VECTORMAP. The initial solution of the heavy-atom derivatives for the DNA-containing crystals was provided both by interpretation of difference Pattersons and by difference Fourier syntheses using phases from the non-DNA-containing crystals. The phases used for locating heavy atom positions derived initially from SIR or MIR calculations, but later corresponded to those improved by solvent flattening. Shown are the results of iterative improvement of the initial derivative site positions and relative occupancies using heavy atom refinement in the presence of the solvent-flattened phases²⁰. The relative occupancy parameters (occ) are on an arbitrary scale. The heavy-atom scattering contributions were calculated from appropriate single atom scattering factors even for the heavy-metal cluster derivatives TAMM and PIP; isotropic thermal parameters of $B = 20$ Å² were used and not refined. The parameters used for automatic mask generation during solvent flattening¹⁸ were (for both crystal forms): solvent fraction = 65%; the empirical constant $S = 0.3$ and radius = 15 Å. The space group enantiomorph was selected to be $P3_212$ (versus $P3_112$) on matching the electron density in the vicinity of the putative template-primer binding groove with models for right-handed DNA and the HIV-1 RNase H domain¹² (see Fig. 3).

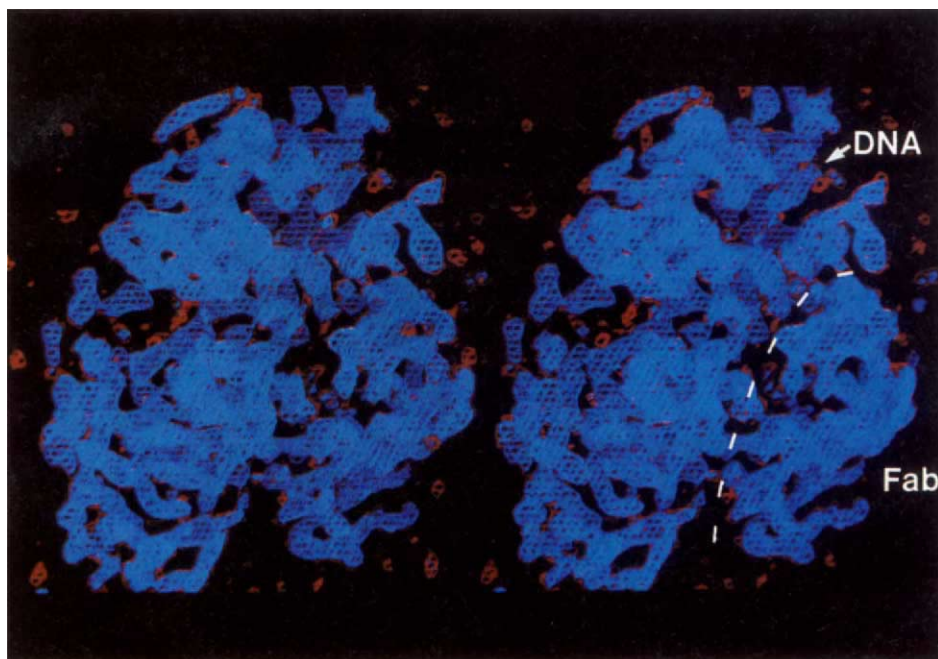
* $R_{\text{merge}} = \sum |I_{\text{obs}} - \langle I \rangle| / \sum \langle I \rangle$. † $R_{\text{deriv}} = \sum |F_{\text{PH}} - F_{\text{P}}| / \sum F_{\text{P}}$. ‡ $R_{\text{centric}} = \sum ||F_{\text{PH}} \pm F_{\text{P}}| - F_{\text{H}}| / \sum |F_{\text{PH}} - F_{\text{P}}|$. § Phasing power = $[\sum |F_{\text{H}}|^2 / \sum (|F_{\text{PH,obs}}| - |F_{\text{PH,calc}}|)^2]^{1/2}$.

infected cells. Reverse transcriptase is the target of the most widely used treatments for AIDS, 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxyinosine (ddI), but resistant strains of HIV-1 arise in patients after a relatively short time^{1,2}. There are several non-nucleoside inhibitors of HIV-1 reverse transcriptase³⁻⁶, but resistance to such agents also develops rapidly⁷. We report here the structure at 7 Å resolution of a ternary complex of the HIV-1 reverse transcriptase heterodimer, a monoclonal antibody Fab fragment⁸, and a duplex DNA template-primer. The double-stranded DNA binds in a groove on the surface of the enzyme. The electron density near one end of the DNA matches well with the known structure of the HIV-1 reverse transcriptase RNase H

domain¹². At the opposite end of the DNA, a mercurated derivative of UTP has been localized by difference Fourier methods, allowing tentative identification of the polymerase nucleoside triphosphate binding site. We also determined the structure of the reverse transcriptase/Fab complex in the absence of template-primer to compare the bound and free forms of the enzyme. The presence of DNA correlates with movement of protein electron density in the vicinity of the putative template-primer binding groove. These results have important implications for developing improved inhibitors of reverse transcriptase for the treatment of AIDS.

We have described procedures for the preparation of crystals of a complex of HIV-1 reverse transcriptase, an Fab fragment,

FIG. 1 Stereoview of the contents of a crystallographic asymmetric unit of the crystals containing a ternary complex of HIV-1 reverse transcriptase p66/p51 heterodimer, Fab28, and 19/18 DNA. Electron density is contoured at 1.0 σ (red) and 1.4 σ (cyan) for an entire crystallographic asymmetric unit in the DNA-containing crystals viewed down the crystallographic 3-fold axis (data and phases used for the calculation are described in Table 1a; drawn using the program TOM/FRODO²¹ on a Silicon Graphics 4D120/GTX IRIS workstation). The overall volume spans 170 Å × 170 Å × 37 Å. The region corresponding to the DNA-binding groove is indicated (shown in the same orientation as in Fig. 3). The density that has been tentatively assigned to Fab28 is also indicated. This region has a reasonably distinct boundary that separates it from the major portion of density (delineated by dashed lines), has dimensions close to those expected for an Fab (~80 Å × 40 Å × 35 Å), and seems to have the expected low α -helical content of an Fab. The largest connected portion of the electron density is interpreted to correspond to the p66/p51 heterodimer; domain boundaries within the heterodimer cannot be assigned with confidence at this resolution. Large solvent channels are present which



account for about 75% of the volume. This is consistent with the unusual observation that dsDNA oligomers can be soaked into preformed crystals grown in the absence of DNA¹⁶.

FIG. 2 Superposition of the electron density maps in the region of the template-primer binding groove for the DNA-bound and DNA-free crystals. The yellow contours represent the electron density computed with data for the non-DNA crystals (Table 1b) and the red contours represent the electron density computed with data for the DNA-containing crystals (Table 1a). The atomic model shown corresponds to an 18-bp B-form DNA. For an explanation of the green contours see Fig. 3. Figures 2 and 3 were displayed using program O (ref. 22).

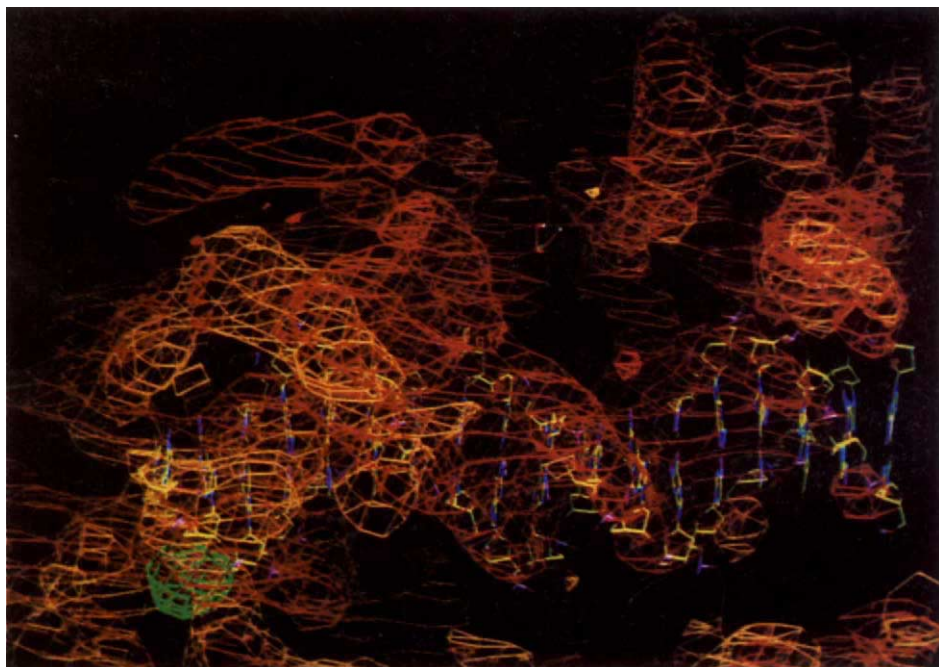
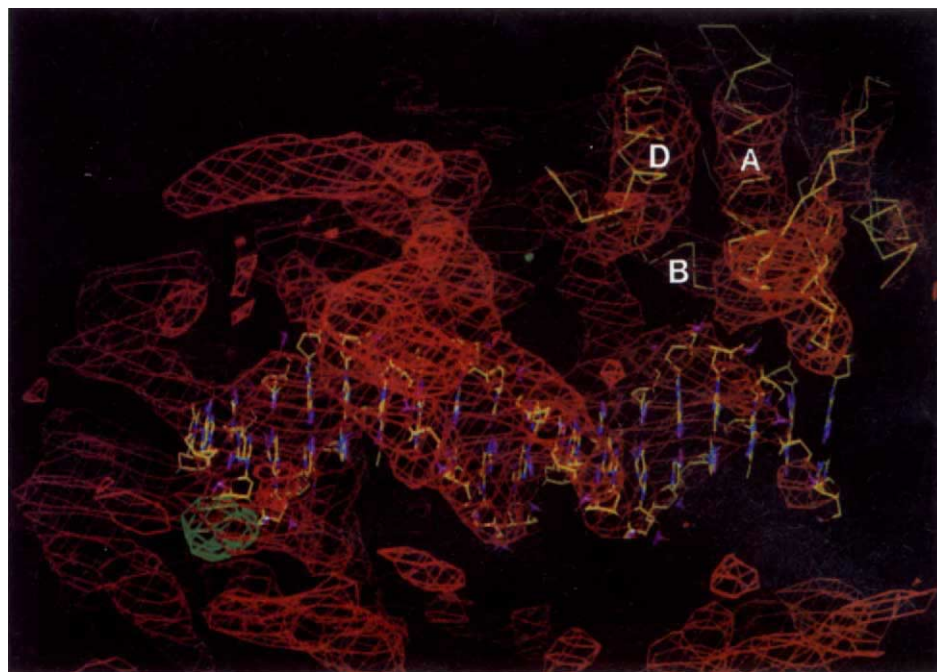


FIG. 3. Relative locations of the dsDNA, the nucleoside triphosphate-binding site, and the RNase H domain in the DNA-containing crystals. Red contours represent electron density in the DNA-containing crystals near the DNA-binding groove. Atomic coordinates in the double-helical density correspond to an 18-bp B-form DNA. The $C\alpha$ backbone (yellow) of the crystallographic model for the HIV-1 reverse transcriptase RNase H polypeptide¹² is superposed with the corresponding density in this map. Alpha helices A, B and D of RNase H are indicated. The green contours of electron density represent a peak in the Hg-UTP derivative difference map relative to native (the only other peaks of comparable significance in the entire asymmetric unit are at sites Hg-A and Hg-B). This may correspond to the active site for DNA polymerization.



and DNA¹⁶. The same crystal form can be grown without DNA. We used a 19-base strand paired with an 18-base strand (19/18) dsDNA with a one-base overhang as a template-primer in which the duplex region corresponds to the sequence of the HIV-1 primer-binding site. The sequence of the 19-base strand is: 5'-ATGGCGCCCGAACAGGGAC-3' where the 5'-A forms the one-base overhang. The complementary strand corresponds to the 18 nucleotides at the 3'-terminus of human transfer RNA^{Lys3} that is used as primer to initiate polymerization by HIV reverse transcriptase⁹⁻¹¹. This complex should resemble HIV-1 reverse transcriptase during DNA-directed DNA polymerization.

The structures of both the DNA-containing and of the DNA-free crystal forms have been determined independently at 7 Å resolution by the techniques of multiple isomorphous replacement combined with solvent flattening (Table 1). An overall view of the asymmetric unit of the DNA-containing crystals illustrates the relative locations of the p66/p51 heterodimer (with subunits of M_r 66,000 and 51,000), the monoclonal antibody fragment Fab28 (ref. 8), and the 19/18-base pair (bp) DNA template-primer (Fig. 1). It is usually not possible to trace the entire course of polypeptide chains in 7 Å resolution electron density maps; however, the dsDNA and α -helical protein regions can be clearly recognized. The dsDNA was initially identified by a difference Fourier synthesis between the two crystal forms using phases from the non-DNA crystals (Table 1b).

A superposition of the electron density maps for the DNA-containing and the DNA-free crystals in the putative DNA-binding region is shown in Fig. 2. The largest differences between the two crystal forms occur in this portion of the structure. The most significant difference is the presence of density corresponding to the dsDNA. There are also indications of significant movement of protein in the surrounding area. Some rearrangement would be required to accommodate the template-primer without steric interference.

Only the DNA-containing crystal form has electron density conforming to atomic models of DNA. This is illustrated in Figs 2 and 3 with an atomic model of an 18-bp B-form DNA. At 7 Å resolution, it is not possible to define precisely the conformation of the 19/18 template-primer. Much of the double helical density can be fit with either A- or B-form DNA. There are protein moieties that are clearly in close contact with the nucleic acid.

The known three-dimensional structure of the RNase H

domain of HIV-1 reverse transcriptase matches well with the electron density adjacent to one end of the 19/18 DNA (Fig. 3). In particular, there is unambiguous matching of the three main helices, A, B and D, of the HIV-1 RNase H domain¹². (The nomenclature of secondary structural elements for HIV-1 RNase H was based on that for *Escherichia coli* RNase H (ref. 13); helix C is not present in HIV-1 RNase H.) In this configuration, the residues of RNase H that chelate the catalytically essential Mg^{2+} would be facing the electron density corresponding to the dsDNA. The relative positioning of the nucleic acid and the RNase H domain is similar to that of models proposed for binding of the *E. coli* RNase H enzyme to an RNA-DNA heteroduplex^{13,14}.

Finally, the 5-mercuriuridine 5'-triphosphate (Hg-UTP) derivative used in phasing the diffraction data (Table 1a) binds to one site in the DNA-containing crystals that is different from the sites to which the non-nucleoside mercurial derivative TAMM binds. This Hg-UTP site (modelled as two distinct heavy atom sites, Hg-D and Hg-E in Table 1a, separated by 6.3 Å) is located near one end of the electron density corresponding to the DNA at the end opposite the putative RNase H domain (Fig. 3). Hg-UTP has the potential for base pairing to the adenine overhang in the 19/18 template-primer, and also for interacting at the nucleoside triphosphate binding site of the polymerization domain of the enzyme. It can be seen from the B-form DNA model shown in the electron density that this Hg-UTP binding site is separated from the RNase H active site by about 15 nucleotides, consistent with biochemical measurements of the distance between the polymerase and RNase H active sites¹⁵. Therefore, the location of this site is likely to correspond to the DNA polymerase active site of reverse transcriptase (Fig. 3). This suggests that biologically relevant complexes of HIV-1 reverse transcriptase with triphosphate forms of nucleoside analogues, such as AZT, ddI and ddC, can be visualized. Determination of the structure at 3 Å resolution, using data collected at the Cornell High Energy Synchrotron Source, is in progress. □

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Three-dimensional structure of a thermostable bacterial cellulase

Michel Juy*, Adolfo G. Amit*, Pedro M. Alzari*†, Roberto J. Poljak*, Marc Claeysens‡, Pierre Béguin§ & Jean-Paul Aubert§

* Unité d'Immunologie Structurale (URA 359 CNRS), Département d'Immunologie, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

‡ Department of Biochemistry, University of Ghent, KL Ledeganckstraat 35, B-9000 Ghent, Belgium

§ Unité de Physiologie Cellulaire (URA 1300 CNRS), Département des Biotechnologies, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

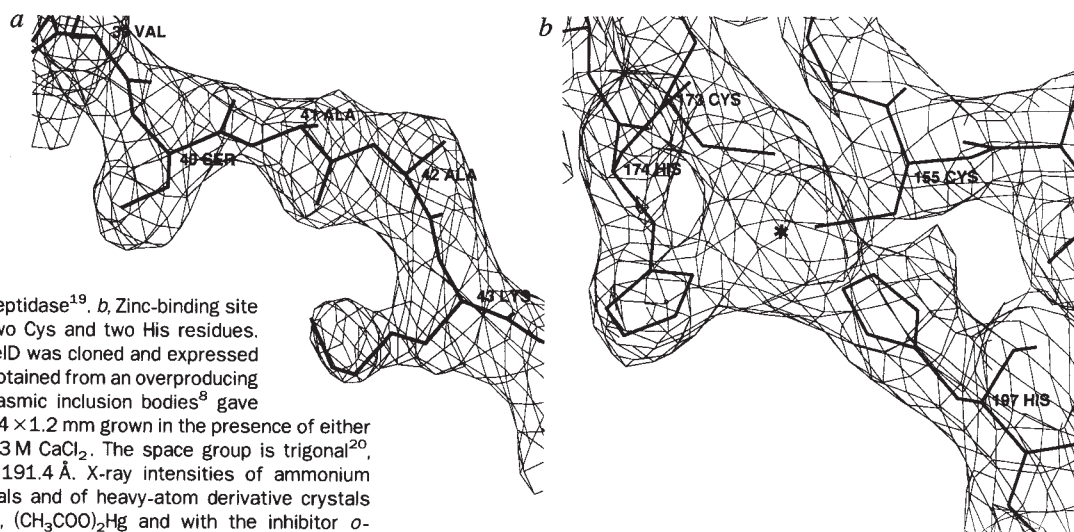
† To whom correspondence should be addressed

CELLULOSIC biomass is recycled by a variety of microorganisms occupying different habitats¹. Studies of their cellulase systems have included the purification of enzyme components, the determination of their enzymological properties² and the cloning and

characterization of their structural genes³. Sequence analysis of more than 70 cellulases permits grouping into seven families corresponding to distinct structural types^{4,5}. The three-dimensional structure of the catalytic core of cellobiohydrolase CBHII from the fungus *Trichoderma reesei* has been reported⁶. Here we show that endoglucanase CelD from *Clostridium thermocellum*, which is representative of a different family of cellulose-degrading enzymes consisting of at least 11 bacterial, fungal and plant endoglucanases^{5,7}, has a globular structure, with an amino-terminal immunoglobulin-like domain tightly packed against a larger catalytic domain. The latter shows a novel protein fold, shaped like an α -barrel of 12 helices connected by loops that form the active site. The structure of a complex CelD with a substrate analogue suggests a mechanism for substrate hydrolysis.

The three-dimensional structure of endoglucanase CelD from *C. thermocellum* was determined by X-ray crystallography. Two forms of native CelD (crystallized with either ammonium sulphate or CaCl_2) as well as that of an inhibitor (*o*-iodobenzyl-1-thio- β -cellobioside)–CelD complex were independently refined at 2.3 Å resolution (Fig. 1). CelD has a globular, slightly elongated shape with rough dimensions of $50 \times 50 \times 70$ Å. It contains two distinct structural domains (Fig. 2a): a small

FIG. 1 Representative regions of the multiple isomorphous replacement electron density maps at 2.8 Å resolution (contoured at 1σ levels). a, Fragment of the N-terminal segment structurally detached from the globular core and stabilized by the interaction of Lys 38 with a neighbouring molecule. The peptide bond between Ala 41–Ala 42 is the possible target of signal peptidase¹⁹. b, Zinc-binding site tetrahedrally coordinated by two Cys and two His residues. METHODS. The gene encoding CelD was cloned and expressed in *Escherichia coli*. The enzyme, obtained from an overproducing clone after extraction of cytoplasmic inclusion bodies⁸ gave crystals measuring up to $0.4 \times 0.4 \times 1.2$ mm grown in the presence of either 0.8 M ammonium sulphate or 0.3 M CaCl_2 . The space group is trigonal²⁰, $P3_121$, with $a=98.9$ Å and $c=191.4$ Å. X-ray intensities of ammonium sulphate and CaCl_2 native crystals and of heavy-atom derivative crystals (obtained with K_2PtCl_6 , $\text{Nb}_6\text{Cl}_{14}$, $(\text{CH}_3\text{COO})_2\text{Hg}$ and with the inhibitor *o*-iodobenzyl-1-thio- β -cellobioside) were measured using a Siemens-Xentronics area detector mounted on a Rigaku RU-200 rotating anode generator and reduced with programs XDS (ref. 21) and the CCP4 software package²². Phasing to 4.5 Å resolution gave a mean figure of merit of 0.66 for the four heavy atom derivatives. A highly contrasted electron density map showed the envelope of one molecule in the asymmetric unit (solvent content ~70%) and allowed phase extension to 2.8 Å using isomorphous replacement and solvent flattening. The polypeptide chain was unambiguously traced from residues 36 to 574 on a PS300 graphics system using program FRODO²³ and a skeletonized version of the map²⁴. To build the atomic model all main chain and β atoms were positioned from a library of refined structures²⁵. After stereochemical regularization, side chains were added and oriented manually into the density. No breaks in the electron density were observed at the 1σ level for the entire polypeptide chain, although some hydrophilic side chains protruding into the solvent were not visible in the map. The initial *R*-factor was 36.8% for 24,310



reflections in the resolution range 8–2.8 Å. After a few cycles of conventional least-squares refinement (energy minimization using program XPLOR²⁶), the *R*-factor dropped to 23.7%. A first round of simulated annealing refinement performed with XPLOR reduced the *R*-factor to 19.4%. Subsequently, least-squares refinement alternating with visual inspection of ($2Fo - Fc$) maps was carried out independently for the two native data sets and for the inhibitor–enzyme complex, gradually extending the resolutions to 2.3 Å; water molecules were added in the last stages of refinement. The number of reflections (6–2.3 Å) used in refinement ($I > 2\sigma(I)$), *R*-factors, r.m.s. deviations in bond lengths and bond angles and the number of added water molecules for the ammonium sulphate, CaCl_2 and inhibitor–enzyme structures are 45,388, 18.8%, 0.014 Å, 3.1°, 216; 41,859, 18.5%, 0.014 Å, 3.1°, 204, and 45,131, 18.8%, 0.015 Å, 3.2°, 226, respectively (further details of structure determination will be published elsewhere).

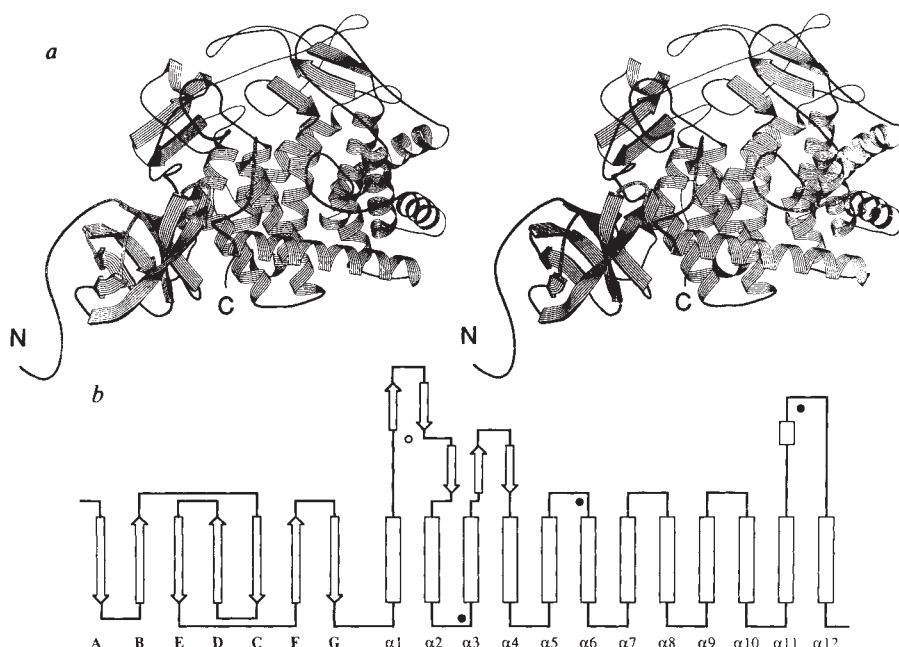


FIG. 2 Three-dimensional structure and topology of CelD. *a*, Ribbon²⁷ stereo diagram of CelD showing the arrangement of α -helices and β -strands in the two domains. The N and C termini are indicated. The catalytic site can be seen as an opening in the upper part of the polypeptide chain folding. β -strands of the N-terminal domain are labelled A to G; the twelve major helices of the α -barrel are numbered sequentially from $\alpha 1$. Polypeptide loops that form the calcium- and zinc-binding sites are indicated by filled circles and circles, respectively.

FIG. 3 Least-squares superposition of immunoglobulin domains (VL in red, CL in blue and CH2 in purple) and the β -barrel domain of CelD (green). The 48 core C α positions matched in individual comparisons were defined as in ref. 28. The loops connecting the β -strands are systematically shorter in CelD than in immunoglobulins, with the exception of the β -hairpin loop between strands D and E. As observed also in the N-terminal domain of the chaperonin PapD protein²⁹ (another example of a prokaryote immunoglobulin-fold lacking a central disulphide bridge), strand D partially belongs to both β -sheets.

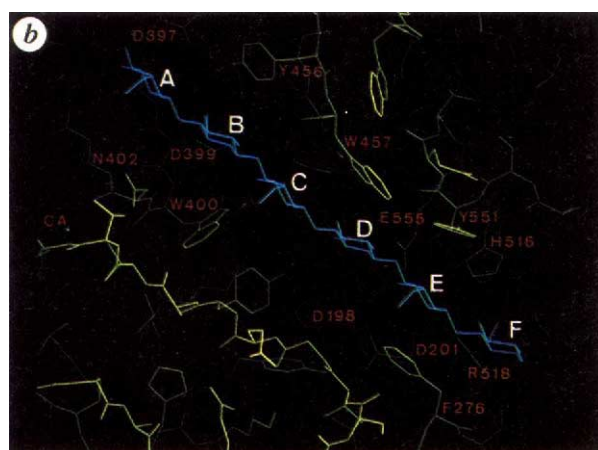
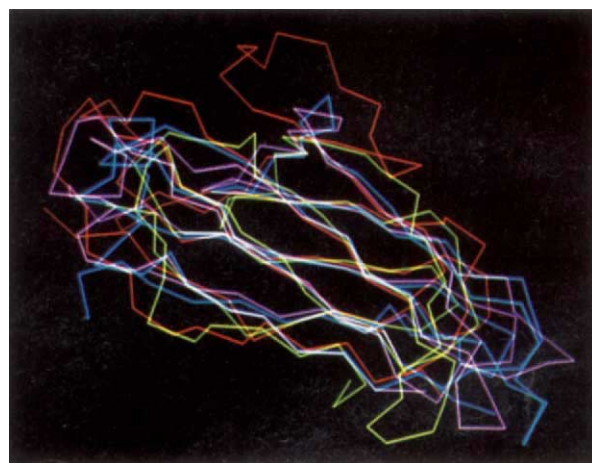


FIG. 4 The active site of CelD. *a*, Partial view of the inhibitor binding site. The difference Fourier map calculated with isomorphous replacement phases at 2.8 Å resolution shows the bound inhibitor, *o*-iodobenzyl-1-thio- β -cellobioside. The iodobenzyl moiety of the inhibitor is bound to a second adjacent subsite, whereas the D-glucosyl unit at the non-reducing end of the molecule is not seen in the electron density map and is thus not represented in the model. The middle region of the long groove is occupied by the N-terminal region of a neighbouring molecule whose Lys 38 can be seen. *b*, General view of the active site with a modelled substrate. Glucosyl-binding subsites are labelled A to F from the non-reducing end of the oligosaccharide. Aromatic and polar residues thought to be important for substrate binding are labelled. The D-glucosyl unit at subsite E is stacked between Tyr 551 and Phe 276. The mean plane of residue Trp 457 is roughly parallel to the oligosaccharide and positioned to participate in substrate binding at subsite D. On the same side of the groove, Tyr 456 can interact with sugar residues at subsites B or C and, opposite, Trp 400 protrudes into the active site and may also interact with substrate residues. The presumed catalytic residues Glu 555 and Asp 201 are close to the glycosidic bond between subsites D and E. His 516 and Arg 518, which are also highly conserved in cellulases of the CelD family, are favourably oriented to interact with polar groups of the substrate at subsites E and F. Towards the non-reducing end a few other polar planar groups are favourably oriented to interact with the substrate: aspartate residues 397, 399 and 401 and asparagine residues 402 and 559.

N-terminal β -barrel closely packed against a larger, mostly α -helical domain. In the latter a long, open groove runs across one face of the molecule. Amino-acid residues preceding Glu 36 (sequential numbering from initiator Met 1; see ref. 8) and beyond Tyr 574, which corresponds to the beginning of a C-terminal region (~60 residues) conserved in many *C. thermocellum* cellulases^{3,9}, are apparently disordered in the crystal structures and have been excluded from the models.

Although CelD contains five cysteine residues of which, even under denaturing conditions, only three react with Ellman's reagent (M.C., unpublished data), disulphide bridges are not observed in the crystal structure. The thiol groups of residues Cys 155 and Cys 173, together with the imidazole rings of His 174 and His 197, form a tetrahedral binding site for a ligand that has been modelled as a zinc atom (Fig. 1b). Three calcium-binding sites were found in the crystal structure, all of them formed by six or seven oxygen atoms from side-chain carboxylates, main-chain carbonyl groups and water molecules. The occupancy and local geometry of these sites is comparable among the three independently refined models and, although they are formed by helix-connecting loops (Fig. 2b), their architecture does not conform to the 'EF-hand motif' found in several calcium-binding proteins¹⁰.

The N-terminal barrel (residues 52–135) contains seven anti-parallel strands, A to G (Fig. 2), forming two β -sheets packed against each other and enclosing a hydrophobic core of aliphatic and aromatic side chains. The overall topology is as in the constant domains of immunoglobulins, although the CelD β -barrel lacks the disulphide bridge between strands B and F, characteristic of immunoglobulin domains. Furthermore, 48 core C α of the CelD β -barrel superimpose with the variable VL and the constant CL-domains of an Fab fragment¹¹ and with the constant domain CH2 of an IgG Fc-fragment¹², with root mean square (r.m.s.) deviations of 1.55, 1.65 and 1.67 Å, respectively (Fig. 3). This structural similarity contrasts with the lack of sequence homology, as only three to four amino acids are identical between the core of each immunoglobulin domain and that of CelD. The CelD β -barrel thus represents a variant of the immunoglobulin fold, structurally intermediate between that of a V and a C domain. The function of this domain is unknown; it is not involved in catalysis (other endoglucanases in the same structural family lack this domain) and probably not in attachment to the cellulosome either, because this interaction seems to be mediated by the conserved C-terminal region present in most *C. thermocellum* cellulases⁹.

The large domain, positions 136 to 574, has twelve major helices, ranging in length from 10 to 24 residues and involving about 40% of the total residues in the subunit (Fig. 2a). These helices are connected following a nearest-neighbour, up-and-down pattern (Fig. 2b) that folds into a right-handed toroidal assembly in space. The arrangement, which represents a novel pattern of folding in globular proteins, is best described as a 'twisted α -barrel', with six inner helices running in roughly the same direction, and six outer helices oriented in the opposite sense. On one side of the barrel, long helix-connecting loops form the substrate-binding site. These loops comprise about half of the total amino acids in the domain and have only small stretches of α -helix or β -strand elements (Fig. 2). A complex network of polar interactions involving protein and metal atoms and several tightly bound water molecules compensate for the lack of secondary structure.

CelD catalyses the hydrolysis of 4'-methylumbelliferyl- β -glycosides of cellobiose and cellotriose, both with liberation of cellobiose. But the catalytic efficiency is almost 30-fold higher for the cellotrioside¹³, suggesting that the binding site is extended. Although in the crystals the long groove running across the helical domain is partially occupied by the signal peptide of a neighbouring molecule, the structure of the inhibitor-enzyme complex confirms that it contains the active site. Indeed, the inhibitor occupies two D-glucosyl-binding subsites of the

groove (Fig. 4a) and no significant steric conflicts are found in modelling four additional sugar residues along the groove (Fig. 4b). Furthermore, the cellulose chain can extend from both extremities of the hexasaccharide without hindrance. Starting from the non-reducing end, the inhibitor occupies the last two subsites, labelled E and F in Fig. 4b. As observed in other carbohydrate-binding proteins^{6,14}, several aromatic and polar residues contribute to the accessible site and are favourably positioned to interact with cellulose.

Site-directed mutagenesis and chemical modification techniques indicate that His 516 lies in the active centre¹⁵ and Glu 555 participates directly in catalysis¹⁶. Furthermore, mutations of Asp 198 and Asp 201 reduce the k_{cat} of the enzyme by >1,000-fold¹⁶, which is compatible with direct participation of these residues in catalysis. Indeed, the side chains of these four residues face the substrate (Fig. 4b).

The structures of the active sites of CelD and of lysozyme are somewhat similar and suggest that in the two enzymes, the scissile glycosidic bond lies between the D and E subsites, with the catalytic residues positioned on both sides of it. Lysozyme Glu 35 and CelD 555 carboxylates occupy closely matching positions relative to the substrate¹⁷, suggesting by analogy that Glu 555 acts as a proton donor in the reaction. But the C α atoms of the two residues are located at about 10 Å from each other as the local conformation of the main chain is different in the two enzymes. Among the two essential aspartate residues identified by site-directed mutagenesis, Asp 198 is far (~8 Å) from the labile glycosidic bond of the substrate; furthermore, the adjacent His 197, which is involved in zinc binding, pulls it away from the active site (Fig. 1b). This suggests that Asp 201 is the nucleophile participating in the acid-base-catalysed reaction. But in contrast to Asp 52 of lysozyme, the carboxylate group of Asp 201 is too far away to interact directly with a positively charged reaction intermediate. Instead, the pocket occupied by the ϵ -amino group of Lys 38 from a neighbouring molecule in the crystal could accommodate a nucleophilic water molecule between Asp 201 and the substrate. Thus, the different positions of the nucleophile in lysozyme and in CelD would reflect the stereospecificity of the reactions catalysed by the two enzymes. Hydrolysis by lysozyme proceeds by double substitution with retention of configuration. Hydrolysis by CelD leads to inversion¹⁸ and is likely to proceed by a single substitution mechanism involving attack of the glycosidic bond by Glu 555 acting as a general acid catalyst and, on the other side of the substrate, a water nucleophile whose ionization is promoted by Asp 201. □

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Capillary electrophoresis: the promise and the practice

Ira S. Krull and Jeff R. Mazzeo

The field of capillary electrophoresis has undergone rapid development over the past 10 years. Many advances have been made, but there are still some problems that must be addressed before the technique can reach its full potential.

In the past 10 years, capillary electrophoresis (CE) has become an area of considerable interest in several areas of analysis, but especially for biopolymers. Starting with a few papers in the late 70s and early 80s, the area has now grown to hundreds of papers and presentations each year for the past 4–5 years, several international symposia dedicated to CE, short courses, university-oriented and required graduate courses, plus special symposia at most major analytical chemistry/biochemistry meetings. Two texts appeared between 1987–1990 (refs 1,2), and several more are due to appear in the next year or so. Capillary electrophoresis has become a hot research and development area in analytical chemistry, especially with regard to capillary modifications, buffer additives, applications, instrumentation development and theory. Its applications for peptide mapping, protein purity checks, nucleic acid purity determination and identification and, perhaps, human genome mapping, seem endless.

Capillary electrophoresis is, in effect, another form of electrophoresis, albeit in capillary format, offering improved opportunities for online detection, identification and, perhaps, quantitation. The schematic of a typical CE instrument is shown in Fig. 1. Fused-silica capillaries with typical inner diameters of 10–100- μm i.d. are used as the separation channel. They may or may not be coated on the inner wall. A high-voltage power supply capable of delivering up to 30 kV is the main driving force for separation. In free-solution CE (or capillary zone electrophoresis, CZE), capillary gel

electrophoresis (CGE) and micellar electrokinetic capillary chromatography (MECC), the two buffer reservoirs and the capillary are filled with the same background electrolyte. A small amount of sample is introduced into the capillary by either gravity/vacuum or electrokinetic injection. In capillary isoelectric focusing (CIEF), the capillary is usually filled with the sample containing ampholytes to generate the pH gradient. Acid is the anode electrolyte and base the cathode electrolyte. In this case, on-column UV detection is shown, although many other detectors have been used, including fluorescence, electrochemical and mass spectrometry. Several companies now sell CE instruments, including Beckman (Fullerton, California), Isco (Lincoln, Nebraska), Bio-Rad (Richmond, California), Applied Biosystems (Foster City, California), Dionex (Sunnyvale, California), Spectra-Physics (San Jose, California), Europhor (France), Jasco (Easton, Maryland), Waters (Milford, Massachusetts), and others. Second-generation instruments are now appearing, containing such features as fraction collectors (Applied Biosystems) and laser-induced fluorescence detection (Beckman and Europhor). Interfacing with mass spectrometry has also been reported on instruments from Isco and Beckman.

Modes of CE

Capillary electrophoresis comprises CGE, CZE, MECC, CIEF and capillary isotachopheresis (CITP). For each separation mode, the instrumentation is essentially the same, although the buffers used and what is inside the capillary may vary.

Capillary gel electrophoresis. In capillary gel electrophoresis, the basis of separation is usually size, based on sieving effects through the gel matrix. It has found widespread use for nucleic acid separations and SDS-proteins^{3,4}. The gel matrix can either be chemically bonded to the capillary wall or, as is the recent trend, polymer networks (or 'physical gels') can be added to the running buffer^{3,6}. These physical gels have the advantage of ease of renewal and greater stability. An example of a CGE separation is shown in Fig. 2. This separation demonstrates the very high efficiencies and resolutions obtainable with CGE. One application of CGE that is in the early stages of devel-

opment is the separation of carbohydrates/polysaccharides⁷. Many exciting developments can be expected in this area.

Capillary zone electrophoresis. Free-solution CE, or CZE, is probably the most common mode of CE. The separation here is based on the charge-to-size ratio of the analyte. Obviously, the pH of the running buffer plays a crucial role in determining selectivity of separation. Separations of amino acids, peptides and proteins are the strong points of CZE^{8–10}. Figure 3 shows the CZE separation of peptides derived from tryptic-digested cytochrome c. Peptide mapping by CZE is completely orthogonal to reversed-phase HPLC separation, such that separations that cannot be achieved by one method are usually possible by the other. Peptide mapping by CZE is one of the main areas of application for CE. The application of CZE to protein separations is limited by adsorption of proteins onto the silica capillary wall. Many different approaches have been described to overcome this problem, including the use of coated capillary surfaces and buffer additives^{11–13}. To date, however, no one method has been described that can be used to separate all proteins of varying pI in one run with a high degree of efficiency and resolution. However, capillary ion analysis, a form of CZE, is an approach that can now rival separations achieved by ion chromatography¹⁴.

Micellar electrokinetic capillary chromatography. This mode of CE involves adding a micellar agent such as SDS to the CE buffer, which will solubilize hydrophobic analytes and ion pair with oppositely charged ones. Micellar electrokinetic

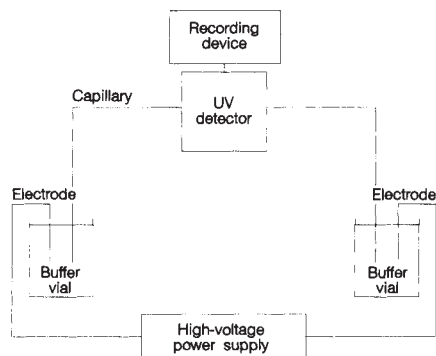


FIG. 1 Schematic of a typical capillary electrophoresis instrument.

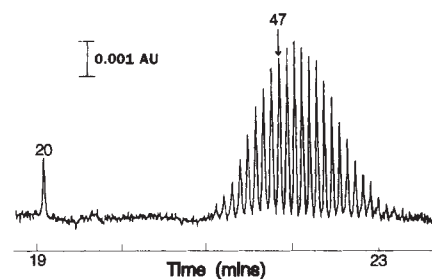


FIG. 2 Capillary gel electrophoresis separation of polydeoxyadenylic acid test mixture, p(dA)40–60 (from ref. 3).

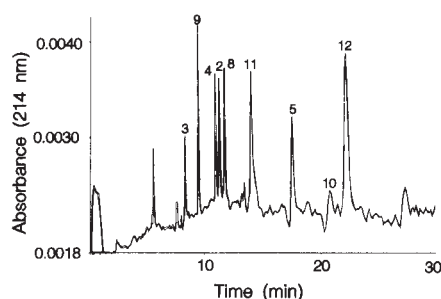


FIG. 3 Capillary zone electrophoresis separation of tryptic peptides of chicken heart cytochrome c (from ref. 9). Chicken cytochrome c peptides: 1, GDIEK (1-5); 2, YIPGTK (74-79); 3, KYIPGTK (73-79); 4, IFVOK (9-13); 5, KTGOAE-GFSYTDANK (39-53); 6, TGOAEGFSYTDANK (40-53); 7, VDLIAY (92-97); 8, MIFAGIK (80-86); 9, TGNLHGLFGR (28-38); 10, VDLIAYLK (92-99); 11, CSOCHTVEK (14-22); 12, GITWGEDTLMEYLENPKK (56-73); 13, GITWGEDTLMEYLENPK (56-72).

capillary chromatography, which has been applied to the separation of numerous small molecules¹⁵⁻¹⁷, has the advantage of being able to analyse serum samples directly due to the solubilizing action of the micelle toward proteins (as in micellar HPLC). One could argue that it is the most mature area of CE, as evidenced by the numerous theoretical and optimization papers that have been written on the subject^{18,19}.

Capillary isoelectric focusing. Capillary isoelectric focusing has been performed in both coated capillaries with salt mobilization²⁰ and uncoated ones with electroosmotic mobilization^{21,22}. To date, it has been applied only to proteins, such as haemoglobin variants and deamidated proteins. However, recently we have been investigating the application of CIEF to peptides to produce peptide maps such as that shown in Fig. 4 (which is the same peptide map as in Fig. 3 except that it is run in the CIEF format). Fewer tryptic peptides are seen, due to the fact that the wavelength is limited to 280 nm, where only tyrosine- and tryptophan-containing peptides can be detected. However, the selectivity is completely different and, more importantly, the migration time of the peptides corresponds to a

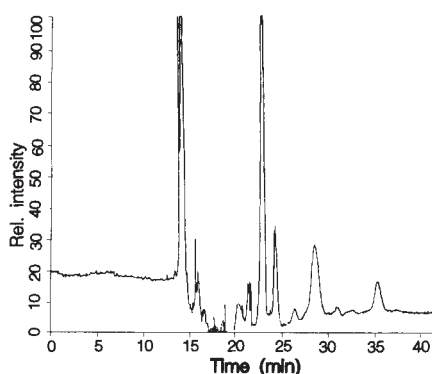


FIG. 4 Capillary isoelectric focusing separation of tryptic peptides of chicken heart cytochrome c.

known property of the peptide — its pI. We foresee peptide mapping by CIEF to be an interesting and potentially useful area of CE.

CE versus HPLC

It would appear that the real strength for CE remains that of conventional electrophoresis, namely the separation of charged species such as nucleotides, nucleic acids and, of course, proteins, peptides and amino acids. Capillary electrophoresis is unlikely to replace current methods of amino acid analysis by HPLC, especially when automated, on-column derivatization methods are possible pre- or post-column in HPLC. However, the greatly improved efficiencies, plate counts and resolutions possible for polysaccharides, polynucleotides, nucleic acids, peptides and proteins, suggest that CE may be here to stay for these important classes of analytes. Rather than replacing HPLC methods, it is more likely that the information that can be obtained by CE will complement, at least in qualitative terms, that which can be obtained by various HPLC modes. Quantitation in an 'absolute' sense (that is, how much of a given peptide or protein is present in a sample) appears more feasible by HPLC rather than CE methods²³. This could be a serious drawback to the future of CE for biopolymer quantitation. While it is true that low molecular weight species, such as drugs or ionic analytes, can be quantitated using both standard addition and/or internal standard techniques, much less has been demonstrated for larger molecular weight species, especially peptides or proteins²³. Though there have been many papers that describe quantitative aspects of CE for proteins and peptides, virtually none of these has really shown that absolute quantitation is yet possible. It has always been the case, however, that conventional electrophoresis was, at best, semi-quantitative and/or qualitative in nature. It may, therefore, not be too surprising that CE may also lack strength in absolute quantitation of large molecular weight, ionic analytes.

Interfacing with MS

Use of capillary electrophoresis in qualitative purity checks, protein/peptide identification, peptide mapping, and interfaced with mass spectrometry (MS) are areas where CE has considerable potential. Of particular interest, is the ease with which numerous forms of CE can be interfaced with MS in its many forms^{24,25}. All of the areas of importance for protein identification — peptide mapping, amino acid sequencing and molecular weight determinations — are now almost as commonplace in CE-MS (using electrospray, ion-spray and continuous fast atom bombardment (FAB) interfacing) as they are for microcolumn HPLC-MS. Although, at present, the cost is somewhat prohibitive, commercial systems (modular or integrated) for performing routine CE-MS applications for biopolymers such as proteins are just over the horizon. It is not as

clear whether aggregates can be detected using HPLC/CE-MS methods, but this may soon be possible using certain forms of FAB. Carbohydrate mapping, glycopeptide mapping, glycoprotein analysis, areas that have been very difficult to accomplish by MS alone, will now become routine using MS in combination with CE.

Quantitative aspects of CE

One of the main problems in CE is the determination of flow concentration levels of an analyte in a sample. Although the mass detection limits with CE are quite outstanding (sub-attogram), concentration detection limits are not as impressive, due to the small injection volumes used. Approaches must be developed that will address this issue. Including automated sample pretreatment and post-column analyte derivatizations or manipulations as part of the CE instrument have also proved difficult to realize. However, there are developments in the pipeline that would allow sample preconcentration on a solid-phase extractor in the capillary entrance, sample stacking, a combination of CITEP and CZE and other variations to enhance/lower analyte detectability^{26,27}. The combination of sample preconcentration and solid-phase derivatization within the entrance to the capillary, perhaps together with laser-induced fluorescence detection, may yet provide the needed levels of detection and sensitivity²⁸.

Capillary coatings and additives

Another issue concerns the capillary itself and the merits of using uncoated versus coated, additives versus coatings, temporary versus permanent gels, pH modifiers, amphipathic polymers, zwitterionic additives and polymeric polysaccharide additives. The problems with a changing capillary surface that lead to analyte adsorption-desorption and loss of recovery may be overcome by additives or capillary coatings. To date, a large number and variety of capillary coatings and additives have been described for any number of specific separations. In the same way that there are now large numbers of capillary columns for gas chromatography and packed columns for HPLC, so too is there likely to evolve a wide array of capillary modifications, permanent coatings or temporary additives to suit different applications and modes of CE.

Strengths of CE

Capillary electrophoresis potentially is a very powerful and useful method of analysis, especially when small amounts of sample are available or the analyte is present at very low mass levels. For those samples that are limited in volume (microlitre/nanolitre amounts) and at low mass concentrations, CE may offer the only hope for successful analysis. Various laser-based detection methods have been developed, and these provide extremely good mass detectability, especially when combined with pre- or post-

column derivatizations. However, on-column reaction chemistry seems much more problematic in CE, especially on a routine basis, although apparatus and techniques for such an approach have been described²⁸.

Although CE offers many opportunities, it is not clear whether or not it will replace existing chromatographic and/or electrophoretic methods of analysis for many species. What is more likely, is that it will provide complementary approaches for identification and analysis, especially qualitative ones, which will be useful and practical. These may be its strongest points. □

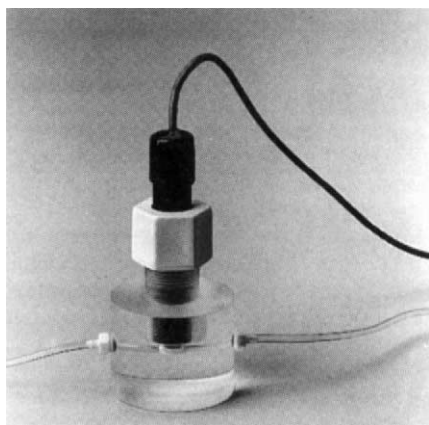
Ira S. Krull and Jeff R. Mazzeo, Department of Chemistry and the Barnett Institute, 341 Mugar Building, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115, USA. This research was supported by a grant and donations of capillary electrophoresis instrumentation from Isco, Inc. We thank K. Patt, J. Algaier, R. Allington, M. Gurkin and J. Tehrani. For more information, fill in reader service number 100.

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Chromatography currents

New product ideas for the chromatographer — columns for environmental analyses and a compact LC system for biomolecule purification.

THE DO-166FT **micro flow-through oxygen probe** from Lazar Research Laboratories is designed to measure very small amounts of dissolved oxygen (*Reader Service No. 101*). The probe, which hooks up



Lazar's micro flow-through oxygen probe measures dissolved oxygen.

directly to any standard pH/millivolt meter or strip-chart recorder, can be used for in-line monitoring of dissolved oxygen in bioreactors, organ perfusion, cell culture, oxygen consumption studies, flow-injection analysis, HPLC and water analysis. Lazar supplies the probe complete with a pO₂ electrode, micro flow cell and elec-

tronic signal conditioner. It has an internal volume of only 120 µl and can be attached to standard sylastic-type tubing or microbore Teflon tubing.

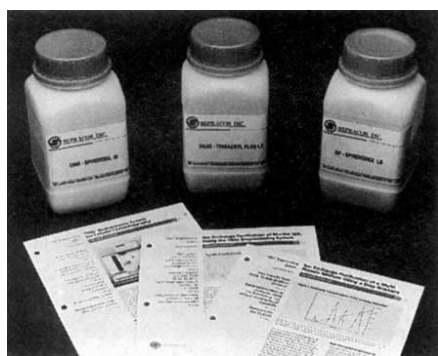
Data Translation announces the DT2804 **chromatography board**, a high-resolution, fast (PC AT-compatible) data acquisition board for capillary gas chromatography, capillary zone electrophoresis and HPLC applications, which is designed to replace traditional hardware integrators (*Reader Service No. 102*). The DT2804 uses an analog-to-digital converter capable of resolving one part in one million (20-bit resolution) at variable integration rates. Up to four different detectors from up to four completely independent chromatographs can be monitored simultaneously. The sampling rate is variable from 1 to 75 Hz. Data Translation says that 'noise' is a mere 0.7 µV RMS at 1 V full-scale or 1 µV RMS at 2 V full-scale. In addition, autocalibration ensures accuracy over time (sample-to-sample consistency) without requiring external calibration equipment. On-board memory and a microprocessor allow the DT2804 to operate independently of the PC AT, simultaneously collect and analyse data and generate reports. Data Translation says it will start shipping the \$1,595 (US) chromatography boards at the end of the month.

Columns and packings

Go green with Shandon's Hypersil Green Series of HPLC columns — specially tailored **alkyl-bonded HPLC phases** that can be used to perform a wide range of environmental analyses (*Reader Service No. 103*). Produced in green hardware to differentiate them from more conventional columns, the Hypersil Green Series are designed to offer exceptional selectivity for polyaromatic compounds (normally difficult to separate with conventional HPLC), normal-phase separation of polyaromatic hydrocarbons (PAHs) in complex nonpolar mixtures such as crude oil or petrochemical fractions, and other common pollutants such as phenols and phthalates produced by the plastics industry, as well as fungicides, herbicides and pesticides. Shandon says that commonly occurring pollutants in river waters and outflows can easily be traced using the Green columns. For example, in the separation of uron herbicides, it is possible to detect 5 ng at the 50-p.p.b. level and, using gradient elution, produce a chromatogram with clearly resolved components and good peak shapes.

Sepracor has introduced a new family of process-scale **chromatography gels** for high-flow, low-pressure ion-exchange chromatography (*Reader Service No. 104*). The line-up includes Trisacryl Plus,

Spheredex and Spherosil — each with different chemical properties to provide a wide range of selectivities for bio-molecules. Trisacryl Plus media, which is available in 40–80 μm and 80–160 μm bead sizes, is a hydrophilic, polymeric matrix that offers good mechanical stability, allowing high throughput in low- and medium-pressure applications. Its stability in pH 1–14 allows cleaning in place, depyrogenation and sanitization of the matrix with acids or bases. Stated applications include fractionation of serum proteins and proteins from tissue homogenates, recombinant proteins, and separation of nucleotides and oligonucleotide mixtures. Spheredex media combines the non-compressibility of silica with the ion-exchange properties of an ionizable polysaccharide. Its ionizable dextran moiety provides hydrophilicity, biocompatibility, superior ion-exchange

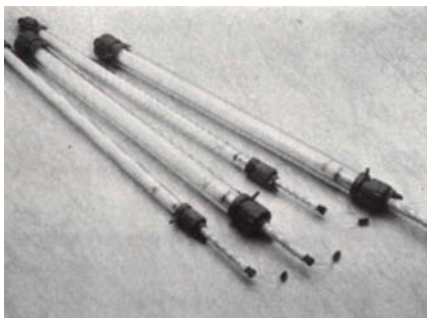


Ion-exchange chromatography gels for every occasion.

kinetics and stability to NaOH. Spheredex is available in 40–80 μm and 100–300 μm bead sizes. Completing the trio is Spherosil media, a combination of both ionic and slightly hydrophobic properties. The media is suitable for the purification of certain macromolecules (such as lipoproteins) that previously have been difficult or impossible to isolate on classical gels. It is available in 40–100 μm and 100–300 μm bead sizes.

■ Pumps and detectors

The HiLoad P-50 pump from Pharmacia Biotech (Europe) is a **biocompatible pump** that has a flow rate range of 0.1–49.9 ml min^{-1} and an operating pressure up to 0.5 MPa (5 bar, 72 p.s.i.) (Reader Service No. 105). The pump, which is a major component in the new HiLoad Plus preparative standard chromatography system, has a wide range of applications in liquid chromatography, microbiology and ferment-



HiLoad columns from Pharmacia for preparative or analytical procedures.

tation. It is intended for use with prepacked HiLoad columns with 'high performance' or 'fast flow' gel media. In addition to pumping aqueous buffer solutions at high flow rates, the HiLoad P-50 pump can also handle viscous biological material such as sonicated suspensions of *Escherichia coli*. The pump is pre-calibrated and is designed to be easy to maintain as a stand-alone pump or as an integrated system component. As part of the same series, Pharmacia offers HiLoad columns, developed for high-resolution preparative work, but which can also be used for analytical separations. The HiLoad columns have a particle size of 34 μm and are designed to provide high resolution at high flow rates. The range includes: 20 columns with 60-cm bed heights for gel filtration and 10-cm bed heights for ion-exchange and hydrophobic interaction chromatography.

Isco's new UA-6 LC detector combines the detector, recorder and fraction-cutting

peak sensor into a compact unit measuring only 28-cm wide (Reader Service No. 106). Its remote, dual-beam optical unit is easily positioned near to the LC column to optimize plumbing. Fifteen interchangeable flow cells are available for low-pressure LC from research-scale to high-flow, HPLC and microbore applications. In addition to the standard 254 nm and 280 nm, fourteen other available wavelengths span the spectrum from 214 nm to 660 nm. Active anti-condensation protection is designed to ensure trouble-free operation in humid coldrooms. The peak sensor controls most fraction collectors, cutting peaks at true beginnings and ends, and the built-in, 10-cm recorder provides event marking along with accurate chromatographic output at a wide range of chart speeds.

Using the latest developments in optics and laser-diode technology, the new **detector for HPLC** from Applied Chromatography Systems, called the Chiramonitor 2000, is designed to provide improved sensitivity and baseline stability (Reader Service No. 107). According to ACS, enantiomeric purity can now be determined (without a chiral column) in just a few minutes, with a precision better than 0.5 per cent. The Chiramonitor should be useful in chiral methods development. As the detector responds with the sign of rotation, sample purity can be determined even if the enantiomers are only partially resolved. The detector responds only to optically-active compounds, which makes enantiomeric analysis possible on even the most complex sample matrix. Specific rotation calculations can now be automated on large numbers of samples using only microgram sample quantities, ACS says. A full supporting software package is also available.

For fast, easy **biomolecule purification** with high bioactivity, Millipore recommends its compact ConSep LC 100 system (Reader Service No. 108). The system is intended for ion-exchange chromatography, affinity chromatography, hydrophobic

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Compact ConSep LC 100 system.

interaction, desalting, concentration and other chromatographic procedures used in the purification of proteins, DNA, peptides and other biomolecules. It is compatible with Millipore's line of MemSep chromatography cartridges and is optimized for membrane convective liquid chromatography, which utilizes high flow rates (50 ml min⁻¹) with accurate gradients. The ConSep system can also run soft gels and smaller preparative columns. The system's 'autoblend' capability provides four-buffer blending, which, Millipore says, is useful in ion-exchange work and methods development. The ConSep system incorporates a pump, injector, multiple-wavelength detector, controller, computer and display. Optional accessories include a fraction collector and a chart recorder.

■ Gas chromatography

OPTIC is the new **temperature-programmed injection system for gas chromatography** from Ai Cambridge (*Reader Service No. 109*). No less than six sampling modes can be used, including new off-line injection and thermal desorption facilities. The system can be installed into most models of GC for use with columns up to 0.53-mm i.d. Ai Cambridge says pro-

grammed injection is the preferred method for sampling into capillary columns. The ability to select an optimal temperature regime greatly reduces the dangers of discrimination and decomposition — frequently encountered with direct high-temperature injection. The injector is of inert construction and ohmically heated for improved thermal performance. The injection temperature can be 100 °C below normal, yet, Ai Cambridge says, quantitative sample transfer can still be achieved, a feature that should be of considerable value in the analysis of thermally-labile compounds. Operating parameters are controlled from a free-standing module, which can be interfaced to the host chromatograph via a rear-mounted I/O socket.

Varian has designed the new **GC Star gas chromatography systems**, which combine the rugged reliability of the 3000 series GCs with enhanced detector performance (*Reader Service No. 110*). The company says it has improved the detection capability of its electron capture detector and thermionic-specific detector in nitrogen mode, providing advanced trace-level sensitivity tailored for specific applications. A new, patented ceramic flame tip allows the Varian flame ionization detector to provide peak shape improvement. This, Varian says, eliminates 'tailing' problems, particularly of polar compounds and high molecular weight hydrocarbons. GC Star systems optimize single-point control of multiple methods. It can be done either directly through its easy-to-use tactile keyboard or, remotely, via the PC-based GC Star workstation.

■ Mail-order means

Everything you need for electrophoresis can be found in Hoefer's 1992–1993 **catalogue** (*Reader Service No. 111*). In addition to instruments for vertical, horizontal and two-dimensional electrophoresis,

the catalogue features products for blotting, densitometry, dialysis, sequencing, filtration, fluorometry, gel drying, gradient making and fractionating, and isoelectric focusing. New lines include a direct blotter that separates and blots in a single process, a capillary cuvette for the mini-fluorometer for the quantitation of DNA in microlitre volumes, a pulsed-field electrophoresis unit with a rotating gel tray, an elution instrument designed to eliminate the disadvantages of dialysis membrane and high salt buffer, a bacterial electrode for the company's electroporation system, a casting stand for making bubble-free sequencing gels, UV crosslinkers for Southern and northern blots, transilluminators, and reagents. The 35-page basic techniques and exercises section should be a useful guide, particularly in university laboratories.

The 1992 256-page, full-colour **chromatography users catalogue** from Hewlett-Packard contains a wealth of product information on the latest chromatography columns, laboratory supplies and consumable products (*Reader Service No. 112*). The catalogue should provide a useful source of information on columns for gas and liquid chromatography, syringes, paper, and environmental samples and supplies for GC, HPLC, UV/visible, FTIR, and GC-MS and LC-MS instruments. New product listings include: HP-50+ and phenyl columns; PLOT columns; HPLC columns; deactivated splitless and insert liners; a split vent trap; a GC-MS separator; vials, inserts and caps; a multi-functional vial rack; and syringes. □

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